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IDENTIFYING BIOMARKERS TO PREDICT THE BIOLOGICAL
BEHAVIOUR OF CANINE CUTANEOUS MAST CELL TUMOURS.

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Abstract

This thesis aimed to identify biomarkers which could be used to determine the biological behaviour of canine mast cell tumours. The rationale for this was that there is a relative paucity of objective biomarkers, which can be established without the need to perform an act of surgery. In order to do this, the potential array of biomarkers available were reviewed. It was considered that markers attainable by interrogation of a blood sample may be most appropriate to fulfil the identified need. After positive results in human oncology, the first analyses focused on the relationships between routinely measured parameters, both proteins (the AGR) and leukocytes (the neutrophil to lymphocyte ratio). Blood samples were taken from dogs with mast cell tumours prior to any treatment intervention. The neutrophil to lymphocyte ratio was identified as a biomarker which could identify canine mast cell tumours with an aggressive biological behaviour.

Building a hypothesis from results in both veterinary and human oncology it was thought that further analysis of the serum proteome could identify further biomarkers of interest. Using the same blood samples, one-dimensional serum protein electrophoresis identified that dogs with biologically aggressive mast cell tumours had more significantly increased α -2 protein concentrations than those with LG mast cell tumours. In an attempt to identify which specific proteins were responsible for changes in the proteome between dogs with mast cell tumours and also when compared to health control dogs, two dimensional serum protein electrophoresis was then performed. Twelve proteins were identified in dogs with aggressive mast cell tumours and not in healthy controls. Some of these proteins are acute phase proteins, reflecting the inflammatory nature of this neoplasm. Others are protease inhibitors which may have a role in tumour pathogenesis. This research has identified biomarkers, which with further validation could have a role in identifying tumours likely to have a severe effect on dogs' quality of life and allow prompt treatment. It has also identified areas for future research.

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Finally to my family- to Laura, my parents and sister for their ongoing love and support.

Author's Declaration

I, Michael Macfarlane, declare that this thesis is my own work except when acknowledged. It does not include work forming part of a thesis presented successfully for a degree in this or another University.

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List of abbreviations

1D-SPE	One dimensional serum protein electrophoresis
2D-DIGE	Two-dimensional difference gel electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
2D-SPE	Two dimensional serum protein electrophoresis
AgNOR	Argyrophilic staining of nucleolar organising regions
AGR	Albumin to globulin ratio
APP	Acute phase proteins
DFI	Disease free interval
FAP	Fibroblast activating protein
FNA	Fine needle aspirate
HG	High grade
HPF	High powered fields
IgE	Immunoglobulin E
ITD	Internal tandem duplications
LG	Low grade
MALDI	Matrix-assisted laser desorption/ionisation
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MC	Mitotic count

MCM7	Minichromosome maintenance protein 7
MCT	Mast cell tumour
MOWSE	Molecular weight search
MS	Mass spectrometry
MST	Median survival time
NLR	Neutrophil to lymphocyte ratio
PCNA	Proliferating nuclear cellular antigen
ROC	Receiver operating characteristic
SCGF	Stem cell growth factor
TK	Tyrosine kinase
TSLC-1	Tumour suppressor in lung cancer-1
WHO	The World Health Organisation

1 Introduction

1.1 Mast Cells and Mast Cell Tumours

Mast cells are granular leukocytes which are involved in type I hypersensitivity reactions and are part of the innate immune system (Day and Shultz, 2010). Mast cells arise from multipotential haematopoietic stem cells within the bone marrow and complete maturation within their destination organ (Kitamura and Fujita, 1989). For this reason, mast cells are often normally identifiable in the circulation of healthy animals (Welle *et al.* , 2008). The mast cell's cytoplasm has many granules, containing a number of vasoactive substances including histamine, heparin, eosinophilic chemotactic factor, metabolites of arachidonic acid (leukotrienes) and proteolytic enzymes which are released on activation of the cell (Dobson and Scase, 2007; Welle *et al.* , 2008). Two distinct forms of mast cells, with different properties, are found in the dog, mucosal (found mainly in the intestine and lung) and connective tissue (found in the connective tissues and skin). Mucosal mast cells generally have greater concentrations of histamine, contained in larger numbers of granules and have a longer life span of up to six months, whereas connective tissue cells usually live less than 40 days (Tizard, 2004).

Mast cells express a number of high affinity receptors for the Fc region of immunoglobulin E (IgE). When an allergen (antigen) binds to the IgE, the crosslinking of two IgE molecules results in activation of the cell and massive degranulation (Tizard, 2004; Day and Shultz, 2010), although a lower level or 'piecemeal' degranulation can also be caused by lipopolysaccharides, endothelins, neuropeptides, adenosine, trauma, heat and toxins (Tizard, 2004; Dobson and Scase, 2007). The release of the substances within granules can cause a wide range of both local and systemic effects (Welle *et al.* , 2008). Activation of the mast cell also results in production of an array of cytokines and chemokines which promote a Th2 and/or pro-inflammatory response from the immune system. These include IL-4, IL-5, IL-6, IL-13, IL-16, TNF- α and CCL-3 (Wilson *et al.* , 2000; Wernersson and Pejler, 2014).

Neoplastic transformation of mast cells can occur at many sites. The skin is the most common location in the dog (Blackwood *et al.* , 2012). Subcutaneous and less frequently, primary oral, gastrointestinal, urethral, spinal, laryngeal, salivary gland, pulmonary and nasopharyngeal mast cell tumours (MCT) have been described, (London and Thamm, 2012; Campbell *et al.* , 2017).

1.1.1. Incidence and Prevalence

In a population of insured dogs in the UK, the incidence of MCTs was 126 per 100,000 dogs per year, the second most common cutaneous malignancy. This population was skewed towards younger animals and the figure including dogs of all ages may be higher (Dobson *et al.* , 2002). Within another population of over 100,000 insured dogs in England the prevalence of MCT was 0.27% (Shoop *et al.* , 2015).

1.1.2. Signalment

Certain breeds linked by a common ancestry have an increased relative risk for developing MCTs with Boston terriers, boxers and English bulldogs having a relative risk of 14 of developing MCTs compared to all other breeds (Peters, 1969). Labrador retrievers, pugs, golden retrievers and the mastiff and terrier phylogenetic cluster (White *et al.* , 2011; Warland and Dobson, 2013; Shoop *et al.* , 2015) also have an increased risk; some other breeds have a reduced risk: English springer spaniels, English cocker spaniels, German shepherd dogs, West Highland white terriers, Border collie and cavalier King Charles spaniels (Warland and Dobson, 2013; Shoop *et al.* , 2015). An increased odds ratio for neutered females developing MCTs has been reported (White *et al.* , 2011), although numerous other reports showed no gender predisposition (Welle *et al.* , 2008).

1.1.3. Aetiology

Although the precise genetic aetiology of most MCTs is not known, in recent years, aberrations of the cellular oncogene *c-Kit* and transcribed protein KIT have been implicated in approximately 14-26% of MCTs (Zemke, Yamini and Yuzbasiyan-Gurkan, 2002; Webster *et al.*, 2006; Letard *et al.*, 2008).

KIT is a class III receptor tyrosine kinase (TK) which, in normal cells, is activated by binding of its ligand- stem cell growth factor (SCGF) (Abu-Duhier *et al.*, 2003). KIT is present in numerous normal cell types and activation of the tyrosine kinase leads to proliferation, maturation, migration and survival of normal mast cells and their stem cells (Ma *et al.*, 1999). KIT expression on malignant mast cells was first noted by flow cytometric analysis (London *et al.*, 1996) and dysregulation and constitutive activation in MCTs have been heavily investigated as an aetiopathogenic factor.

Constitutive activation of KIT in MCTs is usually a result of mutation of the *c-kit* proto-oncogene, often within the juxtamembrane coding region (Ma *et al.*, 1999). Numerous *c-Kit* mutations have been identified: deletions, insertions or single base pair alterations (Zemke, Yamini and Yuzbasiyan-Gurkan, 2002).

In a population of 191 dogs with MCT, 32 of 50 mutations detected in the *c-kit* oncogene were in the juxtamembrane domain, within exon 11 (Letard *et al.*, 2008). Other mutations within exons 2, 5, 6, 7, 8, 9, the junction of 9 and 10, 15 and 17, and coding for various other sections of the KIT protein have also been identified (Letard *et al.*, 2008; Takeuchi *et al.*, 2013). In 13 of 47 (27.7%) MCTs with previously identified activating *c-kit* mutations, 8 were internal tandem duplications (ITD) on exon 11 (all of which were different), but the functional significance of a further 21 of these mutations was unknown (Takeuchi *et al.*, 2013).

Given the relatively low percentage of MCT with known activating *c-kit* mutations, other as yet unknown factors must also be involved in the aetiology of MCTs (Blackwood *et al.*, 2012).

1.1.4. Presentation

Cutaneous MCTs can develop at any location and 11-14% of affected dogs present with multiple masses (Mullins *et al .*, 2006). Based on their gross appearance, two distinct cutaneous forms have been reported to exist:

- Well differentiated tumours; slow growing and hairless (Figure 1-1).
- Poorly differentiated tumours; ulcerated, rapidly growing and pruritic (Figure 1-2) (Blackwood *et al .*, 2012).

It can often be difficult to categorise tumours in this manner however as MCTs have an extremely varied appearance (Blackwood *et al .*, 2012; London and Thamm, 2012).



Figure 1-1: Typical appearance of a low grade cutaneous mast cell tumour. There is minimal erythema, oedema and the mass is under 1cm in size. This mass is situated on the dorsum of a Labrador retriever.



Figure 1-2: Typical appearance of a high grade cutaneous mast cell tumour. There is erythema, oedema and ulceration. This mass is situated on the lateral elbow of a miniature schnauzer.

Both local and systemic clinical signs can be associated with MCTs as a result of mast cell degranulation (Welle *et al.* , 2008). The wide range of clinical signs reflect the diverse nature of the granules and their normal function. Examples of local signs include pruritus, erythema, oedema and swelling. Systemic signs include: vomiting, diarrhoea, gastrointestinal ulceration, lethargy, a reduced appetite, paroxysmal sneezing following cutaneous trauma, fever, impaired coagulation due to heparin release and collapse (Howard *et al.* , 1969; Welle *et al.* , 2008; Blackwood *et al.* , 2012).

The activation of H₂ receptors within the stomach causes increased release of acid into the gastric lumen resulting in many of the gastrointestinal signs. Dogs with MCT with gastrointestinal signs refractory to H₂ blocking treatment have increased plasma histamine concentrations (Ishiguro *et al.* , 2003).

Gastrointestinal ulceration was identified on necropsy of 20 of 24 dogs with MCTs (Howard *et al.* , 1969). Up to half of dogs with MCTs have paraneoplastic clinical signs (Welle *et al.* , 2008) and 12 of 14 dogs with a cutaneous MCT and bone marrow spread had systemic clinical signs possibly attributable to degranulation (Marconato and Bettini, 2008).

1.1.5. Diagnosis

Cytological diagnosis of MCTs is accurate, with 96% of MCTs correctly diagnosed (Baker-Gabb, Hunt and France, 2003). Mast cells are identifiable by the presence of metachromatically staining cytoplasmic granules (Blackwood *et al.* , 2012), easily seen with routine haematological stains such as Leishman, but also with rapid, modified Romanowsky-type stains such as Diff Quik™ (Welle *et al.* , 2008). Occasionally, toluidine blue and Wright-Giemsa may need to be used to identify granules (London and Thamm, 2012) particularly for MCTs containing few granules, however, these tumours are also difficult to categorise histopathologically (London and Thamm, 2012).

Investigation of a possible MCT requires definitive diagnosis, variable degrees of clinical staging and identification of any paraneoplastic signs (Welle *et al.* , 2008). If a MCT is diagnosed cytologically and there are either additional negative prognostic indicators or the mass is not amenable to wide excision, it is recommended that the diagnostic protocol is expanded to include full clinical staging (Welle *et al.* , 2008).

1.1.6. Clinical Staging

There are consensus recommendations for whether full clinical staging (evaluation of a cancer patient in order to determine the extent of the local disease and presence of any distant metastasis) should be carried out in an individual MCT case (Blackwood *et al.* , 2012). Full staging prior to surgery is not recommended for MCTs where the tumour appears easily resectable and does not have accompanying negative prognostic indicators. Full staging in MCTs historically involved haematological and biochemical profiles, buffy coat analysis, cytological lymph node assessment, abdominal ultrasound with cytological assessment of liver and spleen, thoracic radiographs and bone marrow biopsy (London and Thamm, 2012).

Buffy coat analysis is no longer recommended since mastocytæmia may occur in dogs with several other clinical conditions. The number of mast cells per buffy coat smear was found to be lower in dogs with MCTs than with other clinical conditions (McManus, 1999).

Thoracic imaging is recommended as a part of complete staging in the most recent consensus document regarding MCT (Blackwood *et al.* , 2012). Since this publication, an evaluation of 230 dogs with MCT and thoracic imaging identified none with thoracic metastasis, although metastatic spread from another unrelated tumour was identified in two cases (Warland *et al.* , 2014). Infrequent thoracic metastases have however been previously identified (Welle *et al.* , 2008). Thoracic imaging may also be useful for evaluation of the intra-thoracic lymph nodes which may drain the skin in the cranial half of dogs with MCT.

Due to the potential for the identification of metastasis, cytological sampling of palpably normal or abnormal draining lymph nodes is currently advocated for cases with a confirmed MCT (Blackwood *et al.* , 2012; London and Thamm, 2012). Abdominal ultrasound with fine needle aspirate (FNA) of the liver and spleen is recommended for cases with nodal metastasis (Blackwood *et al.* , 2012). However, metastatic disease to the liver and spleen can be identified in the absence of nodal metastasis, so there may be a role for abdominal ultrasound in all cases of MCT (Pizzoni *et al.* , 2017). The sensitivity of visible ultrasound changes for detecting splenic and hepatic infiltration of MCT was 42% and 0% respectively in a population of dogs with clinically aggressive MCT (Book *et al.* , 2011). The prognosis for dogs with metastasis to either organ was worse than those without metastasis (Book *et al.* , 2011).

Since normal mast cells may be present within the cytological samples from lymph node, liver and spleen of healthy dogs, there are currently no robust guidelines for diagnosis of metastasis using these methods. Criteria have included the presence of sheets of mast cells; more than two high powered fields containing increased numbers of MCTs; a large number or poorly differentiated mast cells; over 50% mast cells in the sample; several aggregates of mast cells seen and/or if poorly differentiated mast cells are seen (Gieger *et al.* , 2003; Cahalane *et al.* , 2004; Thamm, Turek and Vail, 2006; Stefanello *et al.* , 2009; Baginski, Davis and Bastian, 2014). Others have not documented their diagnostic criteria (LaDue *et al.* , 1997; Thamm, Mauldin and Vail, 1999; Sfiligoi *et al.* , 2005). The University of Pennsylvania Clinical Pathology Service has formulated diagnostic criteria, with 'certain metastasis' defined as: 'On at least one slide, effacement of lymphoid tissue by mast cells, and/or the presence of aggregated, poorly differentiated mast cells with pleomorphism, anisocytosis, anisokaryosis, and/or decreased or variable granulation, and/or greater than five aggregates of more than three mast cells' (Krick *et al.* , 2009). When considering histopathological identification of lymph node metastasis, staging criteria are available in a four-tiered classification system, with advancing stage

found to be a poor prognostic indicator (Weishaar *et al.* , 2014). This grading system has not currently been applied to cytological examination.

1.1.7. Treatment

The diverse biological activity of MCTs has resulted in the routine use of a number of treatment options although surgery, radiotherapy and chemotherapy, either as single treatments or in combination are the usual treatment modalities (Welle *et al.* , 2008; London and Thamm, 2012). Intralesional and systemic corticosteroids, immunotherapy, intraregional deionised water, hypothermia, photodynamic therapy, cryosurgery and electrochemotherapy have also been used (Blackwood *et al.* , 2012; Lowe *et al.* , 2016; Case and Burgess, 2018). Treatments are selected dependent on the presence or absence of negative prognostic factors (London and Thamm, 2012), with treatment algorithms available upon which to base these decisions (Blackwood *et al.* , 2012; London and Thamm, 2012). Paraneoplastic conditions should also be treated, most frequently with antihistaminic agents, blocking both H1 and H2 receptors. Anti-inflammatory doses of corticosteroids are also frequently used in the treatment of paraneoplastic signs.

1.2 Prognostic factors

1.2.1. Non-histopathological markers

In order to obtain a histopathological diagnosis prior to treatment, incisional or punch biopsies are required. This approach is often avoided with mast cell neoplasia due to concerns over mast cell degranulation leading to poor wound healing (O' Keefe, 1990; Dobson and Scase, 2007; Welle *et al.* , 2008; London and Thamm, 2012). Five of twelve biopsy procedures of MCTs resulted in delayed healing, although incomplete resection was not associated with poorer results than complete excision or with removal of a benign neoplasm (Killick *et al.* , 2011).

A number of factors have therefore been examined for their ability to predict the biological behaviour of MCTs prior to attaining a histopathological grade. It is recommended that the presence or absence of these factors is used to guide treatment and staging decisions prior to surgery (Blackwood *et al.* , 2012).

1.2.1.1. *Gross appearance/Clinical signs*

Gross tumour appearance has been suggested to correlate with biological behaviour of MCTs, with rapid growth, local irritation/inflammation, local infiltration/poor demarcation, ulceration, satellite nodules, pruritus and paraneoplastic signs all being indicators of a more aggressive disease course (Thamm, Mauldin and Vail, 1999; Mullins *et al.* , 2006; London and Thamm, 2012). MCTs which have been present for more than 28 weeks have resulted in a better prognosis after removal than those present for less time (Bostock, 1973). Despite this, tumours which appear without aggressive features may still have a high biological activity (Blackwood *et al.* , 2012).

The presence of clinical signs related to the tumour such as gastrointestinal signs, pain, ulceration or oedema also correlate with a less favourable outcome (Mullins *et al.* , 2006).

1.2.1.2. *Tumour location*

MCTs of the scrotum and testes have a significantly shorter disease free interval (DFI) than those in other locations (Sfiligoi *et al.* , 2005) and it is suggested that those in the subungual area and other mucocutaneous sites result in a poorer prognosis (London and Thamm, 2012). MCTs of the muzzle are more likely to be of a higher histopathological grade and to metastasise than those in other locations (Gieger *et al.* , 2003).

1.2.1.3. *Clinical stage*

The World Health Organisation (WHO) Clinical Staging System for MCTs (Table 1-1) (Owen, 1980) correlates with biological behaviour in a number of studies

(Bostock, 1973; Turrel *et al.*, 1989; Gerritsen *et al.*, 1998; Sfiligoi *et al.*, 2005). This may not be applicable to all locations (Gieger *et al.*, 2003) and the presence of multiple tumours (stage III) does not reliably result in a worse prognosis (McNiel, Prink and O'Brien, 2006; Mullins *et al.*, 2006; Murphy *et al.*, 2006; Scase *et al.*, 2006; Thamm, Turek and Vail, 2006; Maglennon *et al.*, 2008). It is thought that multiple canine MCTs are likely to represent *de novo* masses rather than metastatic spread (Amagai *et al.*, 2013).

<u>WHO stage</u>	Definition
<u>0</u>	One tumour in the skin incompletely removed, with no lymph node involvement.
<u>I</u>	One tumour in the skin, with no regional lymph node involvement
<u>II</u>	One tumour in the skin with regional lymph node involvement
<u>III</u>	Multiple large, deep skin tumours, with or without lymph node involvement
<u>IV</u>	Any tumour with distant metastasis or recurrence with metastasis

Table 1-1: World Health Organisation staging guidelines for dogs with cutaneous mast cell tumours (Owen, 1980).

Lymph node metastasis, when treated, also results in similar survival times as dogs with stage 0 disease (Chaffin and Thrall, 2001; Thamm, Turek and Vail, 2006). LaDue *et al .* (1997) showed no association with either lymph node metastasis or clinical stage on DFI or survival in irradiated, incompletely excised MCTs. However, if lymph nodes are evaluated histopathologically, lymph node metastasis is associated with a poorer prognosis (Weishaar *et al .*, 2014).

1.2.1.4. Age

Increasing age is associated with increasing odds of developing distant metastasis (Kiupel *et al .*, 2005) and decreased survival time after MCT surgery (Michels *et al .*, 2002) with dogs over eight years having a reduced median survival time (MST) compared to those under eight (Sfiligoi *et al .*, 2005). These studies did not investigate tumour related death however and co-morbidities are more likely with increasing age.

1.2.1.5. Sex

Male dogs have been reported as having shorter survival times than females (Gerritsen *et al .*, 1998; Kiupel *et al .*, 2005).

1.2.1.6. Breed

Boxers (Bostock, 1973) and pug dogs (McNiel, Prink and O'Brien, 2006) generally have a higher frequency of lower grade tumours despite frequently having multiple MCTs and would therefore be expected to have a better prognosis in general. Conversely, Shar-Peis have been associated with MCT with a higher biological activity and an expected poorer prognosis (Miller, 1995).

1.2.1.7. Cytology

Different cytological characteristics have been used in an attempt to stratify MCTs into groups with different expected biological behaviours, with varying success. With one grading system, only three of six grade III tumours were

correctly classified (Nieto, 2009). However, use of another cytological grading system was more successful, with 94 percent of samples correctly classified when compared to histological grade using cytology alone (Scarpa, Sabattini and Bettini, 2014). This system identified features present in the two-tiered histopathological system, but on a cytological sample, analysing a minimum of 1000 cells. This technique was later repeated with the same proportion of tumours correctly classified (Hergt *et al.*, 2016). Both of these analyses resulted in the misclassification of a small proportion of HG MCT as LG, resulting in the conclusion that this cytological method is inadequate (Hergt *et al.*, 2016).

Another system was created, using either poor granulation of tumour cells or two of four of the following criteria: mitotic figures, binucleated or multinucleated cells, nuclear pleomorphism, or >50% anisokaryosis. This system had 88% sensitivity and 94% specificity for identifying the correct histopathological grade (Camus *et al.*, 2016). Nuclear morphometry can also be used to grade MCTs from a cytological sample with promising results (De Strefezzi *et al.*, 2009), although again this technique is not widely used. Argyrophilic staining of nucleolar organising regions (AgNOR) staining can also be used on cytological specimens and resulted in accurate prediction of histopathological grade (Kravis *et al.*, 1996).

1.2.2. Histopathological prognostic factors.

1.2.2.1. Histopathological Grade

Due to the variability in both the appearance and behaviour of MCTs, prognostic markers are crucial to predict the biological behaviour and guide treatment protocols. Although cytology offers a means of diagnosis, histopathological grade is currently the most objective method for determining the prognosis in a dog with a MCT.

The first mast cell tumour grading system was proposed and evaluated using the following criteria: the ratio between diameter of nucleus and total diameter of

the cell, the frequency of mitotic figures, the degree of cellular pleomorphism, the discreteness of the tumour cells, and the cellularity of the tumour as well as the nature of the intracytoplasmic metachromatic granules (Bostock, 1973). Grade I tumours were the most biologically active tumours and designated as poorly differentiated tumours. However, few objective criteria were used. Grading was based on subjective assessment such as the criteria of 'mitotic figures were seen, sometimes very frequently'. Using this grading system there was a significant difference in survival times in grade III MCT dogs compared to this with grade I or II.

Subsequent to this, a different three-tiered system based on the degree of tissue invasion was used for MCT (Patnaik, Ehler and Macewen, 1984). This grading system designated grade I tumours as having the least aggressive biological behaviour and grade III the most aggressive. This grading system also contains subjective elements and resulted in greater than 50% of tumours being designated as grade II tumours. The majority of dogs (84%) with grade II mast cell tumours treated with surgery alone remained free of mast cell disease, however 5%-56% of dogs die due to their MCT (Patnaik, Ehler and Macewen, 1984; Séguin *et al* ., 2001). This three tiered grading system resulted in significantly different survival times between grades (Patnaik, Ehler and Macewen, 1984).

Using an alternative two-tiered grading system (Kiupel *et al* . 2011), dogs were designated as having high grade (HG) or low grade (LG) MCTs. Dogs with HG MCTs treated with surgery alone had a MST of under four months whereas those with LG MCTs had an MST in excess of two years. Criteria classifying a MCT as HG with this system are over seven mitotic cells per ten high powered fields (HPF) or three or more multinucleated (more than three nuclei) cells in ten HPF or three or more bizarre nuclei per ten HPF.

The usefulness of the newer system has been validated in the original report and independent publications subsequent to its introduction. LG and HG MCTs resulted in a significantly different 1452 and 208 day survival times although

treatments were not standardised between groups (Takeuchi *et al.* , 2013). The two-tiered system has less inter-observer variation and greater concordance amongst pathologists as well as a greater prognostic significance (Kiupel *et al.* , 2011; Vascellari *et al.* , 2013; Sabbatini *et al.* , 2014).

A number of tissue biomarkers have been assessed for their potential use in predicting biological behaviour in MCTs.

1.2.2.2. *Mitotic Count*

Mitotic count (MC) is defined as the number of mitotic cells seen in ten consecutive high powered fields (400x) while mitotic index is defined as the number of mitotic cells divided by the number of cells not in mitosis (Meuten, Moore and George, 2016). Veterinary pathology studies often incorrectly refer to the MC as mitotic index. In this thesis, the term MC will be used, but in references, 'mitotic index' may be used incorrectly. MC has consistently been associated with the biological behaviour of canine cutaneous MCTs, both when considered as a linear scale and when values above and below a threshold value are used.

The two tiered histopathological grading system for MCTs (Kiupel *et al.* , 2011) used MC as one of the elements which can determine the grade. Tumours with a MC of at least seven (evaluating the most mitotically active areas), are considered HG although since other non-mitotic factors can also define HG status, tumours with a MC of less than seven may also be considered HG.

The three tiered histopathological grading system also considers the number of mitoses, but only in a single high powered field (Patnaik, Ehler and Macewen, 1984); Grade I having no mitotic figures; grade II as zero to two mitoses per high powered field; and grade III as three to six. Numerous other factors such as tissue invasion influence this grading system, perhaps explaining why mitotic index was not significantly different between the three grades of cutaneous MCT in one report (Sakai *et al.* , 2002). When the areas of greatest proliferation were

assessed, a cut off value of five mitotic figures per ten high powered fields was predictive of three tiered grade and also outcome for dogs with a grade II tumour (Romansik *et al .*, 2007).

When high power fields were selected at random, there was no significant difference in survival between dogs with a MC of 0-4 and those with a count of 5-10, although the risk of MCT related death was increased in those with a count above 10 (Bostock *et al .*, 1989). A cut off value of five for MC was predictive of all-cause mortality within the study period, although the method of assessing the MC was not reported (Vascellari *et al .*, 2013)

When fields were selected according to those with highest mitotic rate, MC was strongly predictive of survival, mast cell related death and/or development of metastatic disease leading to proposals for both a two tiered (zero to five and greater than five) and two differing three tiered (zero, one to seven and greater than seven; zero or one, two to seven and greater than seven) systems (Romansik *et al .*, 2007; Elston *et al .*, 2009; van Lelyveld *et al .*, 2015) to predict behaviour. A two tiered MC system with a cut off value of five was highly specific (96%) but insensitive (32%) for MCT related death while a cut off value of two was 80% specific and 76% sensitive (van Lelyveld *et al .*, 2015).

The lack of agreement amongst pathologists as to the optimal stratification system and method of assessing MC result in a very confusing picture for the clinical practitioner. This is further confounded by studies which report different prognostic indices and differences as to whether dogs given adjuvant treatment are included in survival analyses. It is however evident that MC is a measure of biological activity and is likely to be a prognostic factor.

1.2.2.3. *KIT*

KIT is the protein expressed by the *c-kit* oncogene. It has been hypothesised that due to its role in tumour development, differences in the staining pattern of KIT between grades of tumour might predict survival of dogs with MCTs. In less

biologically active tumours overproduction of ligand of KIT (stem cell factor) by different cells could induce tumour formation by overstimulation of KIT. In more biologically active tumours, mutations of *c-kit* could lead to constitutive activation of the receptor (Reguera *et al.* , 2000).

The manner in which the KIT protein is distributed in the cell on immunohistochemical staining has been formally described as three distinct patterns; a membrane-associated staining (type I) also found in non-neoplastic mast cells; a focal to stippled cytoplasmic staining with decreased membrane-associated staining (type II); and a diffuse cytoplasmic staining (type III) (Kiupel *et al.* , 2004). Tissue microarray was performed to quantify expression of KIT protein, but this was not associated with KIT protein localisation (Webster *et al.* , 2006). An intracytoplasmic pattern (type II or III) is associated with a worse outcome and greater propensity for tumour recurrence after surgical resection suggesting that the cytoplasmic form may contain activating mutations, which confer a more aggressive phenotype (Webster *et al.* , 2004; Patruno *et al.* , 2014). Type III KIT staining pattern was associated with a shorter DFI and overall survival in dogs with MCTs treated with surgery and chemotherapy with or without radiotherapy treatment (Webster *et al.* , 2008). A correlation between KIT staining and prognosis has not been found universally (Bergman *et al.* . 2004).

Mutation of c-Kit has been linked with biological behaviour of Patnaik graded MCT. However, the presence of *any* activating mutation was not related to overall survival time, suggesting that different mutations may result in different levels of activation of the tyrosine kinase depending on their location (Takeuchi *et al.* , 2013). Four of six grade III MCTs contained mutated c-Kit; eight of 58 grade II tumours showed juxtamembrane mutations of c-Kit while none of twenty-four grade I tumours were mutated. Duplications were most often associated with higher grade tumours (Zemke, Yamini and Yuzbasiyan-Gurkan, 2002). In contrast, no correlation was found between c-Kit mutational status and tumour grade using the two tier grading system (Takeuchi *et al.* , 2013).

ITDs on exon 11 (juxtamembrane region) of c-Kit have been consistently associated with a poorer prognosis, significantly shorter MST and DFI, an increase in MCT recurrence after surgery, and MCT related death. ITDs on exon 11 were found only in grade III neoplasms or were predictive of histopathological grade and were also associated with an intracytoplasmic KIT immunohistochemical staining pattern (Webster *et al.* , 2006, 2008; Takeuchi *et al.* , 2013). Presence of ITDs was not an independent prognostic factor in a multivariable model, but the number of cases was low (eight) (Takeuchi *et al.* , 2013). ITDs affecting exon eleven may therefore be the most functionally significant *c-kit* mutations in MCTs.

Phosphorylated (activated) KIT identified using immunohistochemistry may be a prognostic determinant in canine mast tumours (Thompson *et al.* , 2015). This was substantiated by a further study (Morini *et al.* , 2004) however in spite of this, c-Kit score was not found to correlate with mast cell grade (Bergman *et al.* , 2004).

1.2.2.4. Ki67

Ki67 is a nuclear protein involved in the transcription of ribosomal RNA, expressed during the cell cycle but not in cells which are not cycling (Bullwinkel *et al.* , 2006). The Ki-67 index is therefore a measure of the proportion of cells in cell cycle i.e. the proliferation rate (Vascellari *et al.* , 2013). Ki-67 index correlates well with bromodeoxyuridine uptake which is considered an accurate index of proliferation indicating Ki67 can also be considered a 'gold standard' proliferation marker (Sakai *et al.* , 2002). This marker is often reported as a percentage of cells with positive immunohistochemical staining (Scase *et al.* , 2006), although some report it as positive nuclei in 1000 cells (Abadie, Amardeilh and Delverdier, 1999). Ki67 staining has been examined extensively in MCTs using a variety of assessment methods (Abadie, Amardeilh and Delverdier, 1999; Sakai *et al.* , 2002; Scase *et al.* , 2006; Maglennon *et al.* , 2008; Vascellari *et al.* , 2013; Berlato *et al.* , 2015; van Lelyveld *et al.* , 2015).

A Ki-67 score above a designated threshold has been identified as an independent prognostic variable for dogs with cutaneous MCTs (Abadie, Amardeilh and Delverdier, 1999; Vascellari *et al.* , 2012). A high Ki-67 (using tumours assessed by different laboratories- but using each laboratory's cut-off value) is a sensitive predictor for MCT related death, but is not specific (van Lelyveld *et al.* , 2015). When animals with a low MC (<2) were evaluated, Ki-67 was found to be predictive of survival, although mortality rate for dogs with low MC, high Ki67 MCT was less than 20% (van Lelyveld *et al.* , 2015). Dogs with a high MC and a low Ki-67 were found to have a lower mortality rate than those with a high MC and high-67 (van Lelyveld *et al.* , 2015). Despite this, Ki-67 is not as useful a negative prognostic factor as a high histopathological grade (three tiered system) and in a different population of dogs, did not have additional benefit to MC- which can be attained with no additional cost (Berlato *et al.* , 2015).

Ki-67 proliferation index is significantly different between grade I and II and also between grade II and III MCTs (Sakai *et al.* , 2002). Ki-67 can also successfully stratify grade II tumours (three tiered grading system) into those with a higher and lower biological activity, which had previously been a problem (Abadie, Amardeilh and Delverdier, 1999; Scase *et al.* , 2006; Maglennon *et al.* , 2008; Berlato *et al.* , 2015). This application has been made less useful by the two tiered grading system, which is now in widespread use. However, Ki-67 expression correlates with Kiupel's grading system as well as survival, using a Ki-67 threshold of 10.6 after receiver operating characteristic (ROC) curve analysis (Vascellari *et al.* , 2013).

A flaw in many immunohistochemical biomarkers, but particularly Ki-67, is that the quantitative method of assessment and cut-off-value are not standardised between pathologists. This suggests a need for standardisation, or a requirement for each laboratory to calibrate its own cut-off value.

1.2.2.5. *Proliferating nuclear cellular antigen*

Another proliferation marker explored in relation to prognosis of MCTs in dogs is proliferating nuclear cellular antigen (PCNA), a member of the DNA sliding clamp family (Scase *et al.* , 2006). The concentration of this protein increases markedly in the S or synthesis phase of cellular division (Bravo and Macdonald-Bravo, 1985). The PCNA score in five HPFs was higher in recurrent MCTs compared to non-recurrent MCT (Simoës, Schoning and Butine, 1994). A shorter survival time was demonstrated in tumours with a PCNA score above the median than those with a score below. The number of positively stained cells correlated with MC but performing the stain was more technically demanding than other immunohistochemical methods. This fact combined with later studies that failed to demonstrate a correlation between PCNA score and survival, put into question the utility of this biomarker in canine MCTs (Abadie *et al.* . 1999, Scase *et al.* . 2006).

1.2.2.6. *Argyrophilic staining of nucleolar organising regions*

Argyrophilic staining of nucleolar organising region (AgNOR) describes a technique whereby a silver stain detects sites of transcription for RNA in the cell's nucleus, visible as small nuclear foci (Trerè, 2000).

AgNOR has been assessed as a potential prognostic factor in biopsied and excised MCT (Bostock *et al.* , 1989; Simoës, Schoning and Butine, 1994; Kravis *et al.* , 1996; Scase *et al.* , 2006; Webster *et al.* , 2007). AgNOR count correlates with histopathological grade in some human neoplasms (Bostock *et al.* , 1989) and indicates the rate of cell cycle progression (generation time) in dogs (Webster *et al.* , 2007).

Early work in canine MCT suggested AgNOR count better predicts survival and recurrence than using MC or histopathological grade alone (Bostock *et al.* , 1989). AgNOR count was found to be a good indicator of three month non-recurrence of excised MCTs (Simoës, Schoning and Butine, 1994). AgNOR score

combined with Ki-67 can indicate both generation time and proliferation rate (Webster *et al.* , 2007). When a number of proliferation indices and KIT mutations were investigated in relation to mast cell stage, only AgNOR was found to be significantly associated with a more advanced WHO stage (stage II over stage I) which has previously found to be prognostic (Krick *et al.* , 2009, 2017; Weishaar *et al.* , 2014). However, AgNOR count was not correlated with another proliferation marker (PCNA) (Simoes *et al.* . 1994).

There has not been a standardised method for performing this staining technique and as a result, any laboratory wishing to perform this stain has previously had to perform its own validation. A human standardisation system (Trerè, 2000) was used by Scase *et al.* . (2006) but AgNOR was not able to predict survival independent of the histopathological grade, contradicting Bostock's earlier work and suggesting limited use for AgNOR count as a prognostic biomarker.

1.2.2.7. *Minichromosome maintenance protein*

Minichromosome maintenance proteins are required for DNA replication and are present throughout all phases of the cell cycle but are reduced during differentiation, quiescence and senescence (Berlato *et al.* , 2012). They are considered a more accurate measure of whether a cell is dividing than other cell cycle dependent biomarkers (Laskey, 2005) and multivariate analysis of canine MCTs has shown that expression of minichromosome maintenance protein 7 (MCM7) significantly correlates with prognosis (Berlato *et al.* , 2012). Dogs with a MCM7 score over 0.18 had a MST of 187 days, whilst MST was not reached (at 3668 days) in dogs with a lower score.

1.2.2.8. *Intratumoural microvessel density*

The vascularisation of a tumour is imperative for tumour growth and metastasis (Weidner, 1995). The number of microvessels per square millimetre was found to be an independent prognostic factor along with mitotic count in 32 dogs with MCTs (Preziosi, Sarli and Paltrinieri, 2004).

1.2.2.9. *Serotonin*

Serotonin (5-HT) is a vasoactive substance which has very wide spectrum of actions, including promoting cellular proliferation, differentiation, maturation and migration (Fröberg *et al.* , 2009). MCTs with high biological activity (grade III on the Patnaik grading system), express less serotonin and also less 5-HT_{1A}R (one of its receptors). This is consistent with findings in human neoplasms where its increased expression can indicate a favourable prognosis (Granberg *et al.* , 2000). Correlation to prognostic information was not examined in dogs.

1.2.2.10. *Tumour suppressor in lung cancer-1*

Tumour suppressor in lung cancer-1 (TSLC-1) is a tumour suppressor gene which inversely correlates with MCT Patnaik grade (Taylor *et al.* , 2010). TSLC-1 expression also strongly linked to the behaviour of Grade II MCTs in a cohort of dogs used previously for Ki67 staining (Maglennon *et al.* . 2008). Despite this, results did not reach significance and this can therefore not be reliably used as a prognostic factor without further investigation.

1.2.2.11. *DNA methylation status*

The epigenetic modification of cells can result in or facilitate the progress of cancer by influencing gene expression (Hanahan and Weinberg, 2011). DNA methylation is the addition of a methyl group to a cytosine base that precedes a guanine base. Hypomethylation results in gene expression and hypermethylation results in gene suppression. When these occur on oncogenes and tumour suppressor genes respectively, tumour progression can result. Given the availability and successful application of treatments which can modify methylation in human haematopoietic malignancies (Curran, 2013), this may have therapeutic as well as prognostic implications in veterinary medicine. Therapies targeting methylation have been used successfully with veterinary species in-vitro and in vivo (Hahn *et al.* , 2012; Flesner, Kumar and Bryan, 2014). There are concerns however that targeting hypermethylation of specific genes

can result in hypomethylation of others and thus promotion of metastasis (Szyf, 2008).

Global DNA hypomethylation identified by immunostaining of 5-methyl cytosine is more common in biologically aggressive canine MCTs than well differentiated tumours (Morimoto *et al .*, 2016). This finding warrants further investigation, although the therapeutic targeting of global hypomethylation is difficult to achieve.

1.2.2.12. *Fibroblast activating protein*

Fibroblast activating protein (FAP) is a serine protease which has roles in tumour initiation, progression and metastasis (Giuliano *et al .*, 2017). This protein is produced not by neoplastic mast cells, but by tumour associated fibroblasts and was identified using immunohistochemistry in the stroma of canine mast cells. A score was created based a semi-quantitative scoring system and this correlated positively with mast cells tumours of higher biological grade (both Patnaik and Kiupel grading systems, Ki-67 and MC) (Giuliano *et al .*, 2017).

1.2.2.13. *Others*

Markers which have been studied, but found not to correlate to either grade or prognosis include BCL-2, (Vascellari *et al .*, 2013), VEGF (Amorim *et al .*, 2010), p53 (Jaffe *et al .*, 2000), heat shock proteins 32 and 90 (Romanucci *et al .*, 2017) and tryptase (Kiupel *et al .*, 2004). COX-2 intensity but not immunohistochemical score or extension was not associated with biological behaviour (Vascellari *et al .*, 2013; Gregório *et al .*, 2017).

1.2.3. **Summary of Prognosis**

Currently, clinicians base clinical decisions on the presence or absence of negative prognostic factors (most commonly histopathological grade, mitotic count and Ki67), staging procedures and surgical margins. Many of the pre-histopathological factors appear unreliable, not repeatable or inaccurate over

multiple studies and there is inconsistency in assessment of histopathological markers with regard to measurement technique, adding subjectivity to their use as markers of biological activity.

Identifying novel biomarkers to assist differentiation of LG and HG MCTs could aid prognostication and allow for more evidence-based clinical decision making algorithms to be produced. Serum biomarkers are an attractive prospect in this setting as blood sampling is often indicated for staging or health profiling of dogs with cancer and avoids the potential of complications associated with biopsy.

1.3 Biomarkers

A biomarker is defined as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (Biomarkers Definitions Working Group. 2001). Identification of biomarkers can allow targeted therapy, detection of early stage disease, relapse and remission, assessment of the effectiveness of therapy and the prognosis of disease (Pang & Argyle 2010). In veterinary oncology, proteins, especially acute phase proteins, haematological parameters and also tissue biomarkers have been investigated as biomarkers. All of these may have potential as prognostic biomarkers for MCTs.

Some serum biomarkers have been explored with respect to MCTs, although there is no information on their prognostic significance or ability to predict biological behaviour (Chase *et al* ., 2012). Considering that mast cells within MCTs often release their vasoactive granules leading to systemic clinical signs; there is a reasonable probability that MCTs will affect concentrations of serum proteins that could be identified as biomarkers and that these could improve the clinician’s ability to treat disease appropriately.

1.3.1. Serum Protein Biomarkers

Proteins serve a number of functions in both health and disease states, with the same protein often performing numerous functions. In broad terms, proteins can

be responsible for structure and organisation of cells, involved in cell signalling, membrane transport, catalytic reactions or involved in reaction coupling. Protein is not stored within the body and therefore concentrations are continually regulated and fluctuant, varying markedly in disease states (Engelking 2014).

A number of techniques are available to evaluate known serum proteins. The concentration of individual proteins or larger groups can be determined using spectrophotometry and this is routinely performed as part of a biochemical profile (Engelking 2014). Immunoturbidimetric methods and ELISA can also be used to measure the concentrations of specific serum proteins, with varying accuracy, sensitivity, specificity, cost and availability (Ceron, Eckersall and Mart  nez-Subiela, 2005). The lack of standard samples and quality control schemes are currently limiting more widespread, routine use of many of these methods to measure specific protein concentrations (Eckersall and Moreira dos Santos Schmidt, 2014).

The large-scale evaluation of proteins expressed in a tissue or organism at any moment in time is known as proteomics and this may involve the measurement of a known protein or an attempt to identify the entire proteome. In order to evaluate the entire proteome in serum and identify novel protein biomarkers, the serum must first be separated into protein fractions, which can then be further analysed by mass spectrometry (MS) to identify and also quantify the proteins present.

A problem recognised in identifying serum protein biomarkers stems from difficulty in distinguishing proteins which have leaked into the circulation from tumour cells and those which are blood proteins. Fewer than 1% of discovered cancer biomarkers reach clinical use and this is due in part to commonplace ‘false discoveries’, non-specific biomarkers and differences in the proteome between sexes and in animals of differing age (Kycko and Reichert, 2014).

1.3.2. Quantification of known serum proteins

1.3.2.1. *Albumin Globulin Ratio*

Albumin is produced solely in the liver and has numerous roles, including maintaining plasma oncotic pressure. It is the most abundant serum protein in the dog, representing 35-50% of the total protein concentration in health. Hypoalbuminemia may result from hepatobiliary dysfunction, but can also be a response to other disease- a negative acute phase response. Albumin concentration is typically measured using spectrophotometric methods such as a bromocresol green dye-binding method (Ceron, Eckersall and Martýnez-Subiela, 2005).

Globulins account for the majority of plasma proteins other than albumin and varying concentrations of any combination of hundreds of globulins comprise the globulin fraction of serum. The globulin concentration in dogs is typically calculated by subtracting the albumin concentration from the total solids (typically also measured spectrophotometrically).

The albumin to globulin ratio (AGR) has been used as a prognostic biomarker in human neoplasia. The AGR reflects a negative acute phase response (reduction in albumin concentration) seen in many neoplastic conditions alongside an increased globulin concentration (Stockham and Scott, 2008). Albumin is reduced by its consumption and albumin production is inhibited by chemokines; the concentrations of numerous globulins rise as a result of an inflammatory state- part of the acute phase response (Azab *et al* ., 2013). The combination of these factors into a ratio ameliorates confounding factors present when measuring only one or the other - such as dehydration falsely increasing the serum albumin value (Azab *et al* ., 2013). The AGR has proved to be prognostic for human colorectal cancer, irrespective of whether serum albumin is within the normal reference range (Azab *et al* ., 2013). AGR has been examined in veterinary conditions such as leishmaniasis and feline infectious peritonitis and is a useful monitoring and diagnostic biomarker in these conditions (Amusategui, Sainz and

Tesouro, 1998; Hartmann *et al.* , 2003). AGR was found to be useful at predicting whether a soft tissue mass was either a soft tissue sarcoma or benign soft tissue mass, with an AGR of less than 1.033 giving a sensitivity of 86.4% and a specificity of 53.8% for identifying soft tissue sarcomas. AGR was not useful at predicting the grade of soft tissue sarcoma in the same population (Macfarlane *et al.* , 2015).

1.3.2.2. *Acute Phase Proteins*

Acute phase proteins (APPs) are circulating proteins in which trauma, inflammation or infection result in a concentration change of a magnitude of greater than 25% (Eckersall and Bell, 2010). The function of this response is to promote recovery and survival of the organism in the acute setting in response to stressors. The acute phase response is initiated and propagated by monocytes and macrophages, although mast cell degranulation may also play a role. Numerous cytokines mediate this process and can result in transcription of APPs within the liver. These cytokines include transforming growth factor beta, interleukin-1 and interleukin-6 and tumour necrosis factor (Baumann and Gauldie, 1994). Measured concentrations of APPs are sensitive in detecting inflammation, but lack specificity (Eckersall and Bell, 2010).

Numerous APPs have been examined as biomarkers in veterinary medicine, in various conditions, including neoplasia (Eckersall and Bell, 2010). Dogs with haematological neoplasia had increased concentrations of haptoglobin and C-reactive protein compared to healthy controls or normal reference ranges (Tecles *et al.* , 2005; Atherton, 2013a). Dogs with MCTs have alterations in known APP concentrations, without significant changes in total protein concentration (Chase *et al.* , 2012). C-reactive protein and α -1 acid glycoprotein were increased. Not all known APPs are increased in dogs with MCT however and haptoglobin concentration was not found to be significantly higher than reference value. Additionally, concentration of serum amyloid A was unexpectedly lower than reference value. No difference was identified in acute

phase concentrations between different grades of MCT, when considering the three tiered Patnaik grading system (Chase *et al.* , 2012).

1.3.3. Exploration of the Proteome

1.3.3.1. Mass Spectrometry

MS is a technique employed to measure the mass to charge ratio of biological molecules with the aim of determining their molecular weight as accurately as possible (Koomen, Hawke and Kobayashi, 2005). With regard to proteins, the mass spectrometer identifies the amino acid sequences then proteins are identified based on probabilities when the amino acid sequences are matched to a known database.

The usefulness of MS as a technique results from its sensitivity, being able to analyse peptides with concentrations as low as 10^{-18} molar (Martin *et al.* , 2000). The downside to this sensitivity is that larger samples may overwhelm the capabilities of this device, necessitating prior fractionation and the correct processing of the sample (Gerou-Ferriani, Mcbrearty, *et al.*, 2011).

A mass spectrometer contains a means of ionising the substance to be analysed, a mass analyser which measures the mass to charge ratio of the analyte which has been ionised and a detector that measures the number of ions present at each value (Aebersold and Mann, 2003).

Electrospray ionisation and matrix assisted laser desorption/ionisation (MALDI) are the techniques generally used to ionise solutions and solids respectively (Aebersold and Mann, 2003). Nanoelectrospray rather than electrospray ionisation is now frequently used to create a narrower diameter spray of ions, which improves sensitivity, robustness and reduces the volume of sample required (Gibson, Mugo and Oleschuk, 2009). After ionisation, the particles are analysed by one or more of four classes of mass analysers: quadrupole ion trap; linear ion trap; time of flight and Fourier-transform ion cyclotron resonance (Han, Aslanian and Yates, 2008). All have various strengths and weaknesses and

can also be used in tandem configurations (Lippolis and Reinhardt, 2011). The ion detector can also come in many forms but is typically an electron multiplier which can turn a signal from a single ion into a much larger electrical current (Lippolis and Reinhardt, 2011). Tandem MS (frequently shortened to MS/MS) involves the further fragmentation of detected ions into smaller peptides by collision with an inert gas with further separation by mass (Hunt *et al.* , 1986). This allows better sensitivity for detecting small concentrations of proteins (De Hoffmann, 1996).

Mass spectrometers analyse either entire proteins or enzymatically produced peptides, from which, after fractionation, the entire protein structure is elucidated. Typically enzymatic digestion is performed subsequent to protein fractionation and this sort-then-break approach was first described using two dimensional electrophoresis fractionation (Henzel *et al.* , 1993). Analysis of entire proteins is often limited by the requirement to separate a mixture of proteins into single or simple mixtures and by the inability to analyse large entire proteins due their tertiary structure (Han, Aslanian and Yates, 2008).

Subsequent to the detection of the mass to charge value of a peptide or protein, a protein or gene database is often searched in order to identify the protein (Han, Aslanian and Yates, 2008). Examples include the MASCOT database, which combines three types of searching and produces a score to allow the significance of a result to be assessed (Perkins *et al.* , 1999). In the dog, the identification of proteins has been facilitated by the sequencing of the genome, which means that proteins can be predicted from their amino acid sequence if required (Atherton *et al.* . 2013a).

Mass spectrometers have a role in veterinary proteomics in assisting the identification of potential biomarkers or toxins. Unlike a genome which is generally identical in all cell types, the proteome changes in response to stimuli and can markedly vary from cell to cell and tissue to tissue independent of genomic changes (Han, Aslanian and Yates, 2008). Interrogation of the proteome, therefore allows identification of epigenetic changes and their

consequences which would not be identified using only genomic techniques. Epigenetic changes are widely established as a factor in the development and progression of cancer (Feinberg, Ohlsson and Henikoff, 2006).

MS has previously been used to search for biomarkers of canine cancer (Kycko and Reichert, 2014). Subsequent to exosome segregation, MS was used to identify exosome proteins, which could be used to differentiate dogs with osteosarcoma from control dogs, as well as predicting the stage of the dog's treatment (Brady, 2018). Numerous other studies have utilised MS after other forms of fractionation. These are documented in the following sections of this thesis examining methods of protein fractionation.

1.3.3.2. *Serum Protein Electrophoresis*

Electrophoresis is a technique employed to separate biological substances using an electric field provided by immersed electrodes and a matrix on which the substances can migrate, typically a polyacrylamide gel for protein separation. One dimensional polyacrylamide gel electrophoresis permits analysis of the size, concentration, isoelectric point and purity of proteins (Shi and Jackowski, 1998).

After separation, the gel is stained and densitometric analysis is performed to calculate the relative concentrations within known protein bands. From knowledge of the total protein concentration, the absolute concentrations of protein within each band can then be calculated (Abate *et al* ., 2000).

After electrophoresis six bands of serum proteins are clearly identifiable and these include albumin and five types of globulins- α_1 , α_2 , β_1 , β_2 and γ (Ceciliani *et al* ., 2013). A large scale one-dimensional serum protein electrophoresis (1D-SPE) study of 147 dogs established a reference range for canine serum proteins of 75 healthy control samples (Tappin *et al* . 2011). Despite this, it was suggested that each laboratory should establish their own reference intervals due to differing methodologies between centres.

Proteins in each globulin fraction or band may be up- or down-regulated by disease processes. 1D-SPE is most commonly used as a diagnostic tool in dogs to evaluate hyperglobulinaemia. A monoclonal gammopathy representing a markedly increased production of a single immunoglobulin by B cells is identified as a sharp peak on densitometric analysis. In a population of 147 dogs, in which 1D-SPE was performed mostly due to hyperglobulinaemia, eight had a monoclonal gammopathy attributable to lymphoid neoplasia (Tappin *et al* ., 2011). In another population of eighteen dogs with monoclonal gammopathy, taken from over 3,900 serum protein electrophoresis results, thirteen had lymphoid neoplasia and five had infectious disease which may be present in certain inflammatory and neoplastic conditions (Giraudel, Pagès and Guelfi, 2002).

1.3.3.3. *Two Dimensional Electrophoresis*

Whereas one dimensional serum electrophoresis can fractionate the serum proteome into six identifiable fractions, it generally does not provide adequate separation to allow MS analysis to identify individual proteins. An example of this was shown when the serum proteome of cats with lymphoma was been evaluated, using 1D-SPE as a sorting mechanism prior to MS (Gerou-Ferriani *et al* . 2011). The separation achieved did not result in sufficient protein separation for clear identification of individual proteins.

Therefore, gels which can separate proteins in two dimensions, allowing resolution of up to 5000 protein representing spots on one gel are frequently used (Magdeldin *et al* ., 2014).

1.3.3.3.1. Two dimensional polyacrylamide gel electrophoresis

One of the most commonly used methods of two dimensional serum protein electrophoresis (2D-SPE) is two dimensional polyacrylamide gel electrophoresis (2D-PAGE). The first dimension of separation typically involves one of three methods of finding the isoelectric point and separating by pH. The second

dimension is typically separation by molecular weight (Magdeldin *et al.* , 2014). This technique is considered robust and provides adequate separation to allow mass spectrometric analysis, but has a low reproducibility; a poor ability to detect low concentrations of proteins (especially if other proteins are present at very high concentrations); is low output and labour intensive (Magdeldin *et al.* , 2014).

Numerous studies have scrutinised the canine serum proteome using 2D-PAGE prior to MS. In obese dogs, three proteins were expressed differently in the serum than that of dogs with a normal weight (Tvariionaviciute *et al.* . 2012). The sera were evaluated using 2D-PAGE, before identifying the proteins by MALDI time of flight MS (MALDI-TOF MS). Serum retinol protein four and clusterin precursor were both up-regulated and α 1-antitrypsin was down-regulated. Changes in steroid hormones during the pre-ovulatory period of dogs have also been examined using 2D-SPE (Fahiminiya *et al.* . 2010). 2D-SPE followed by MALDI-TOF MS showed that neuron specific enolase was higher in dogs with meningoencephalitis of unknown aetiology (Nakamura *et al.* . 2012). When considering cancer, 2D-SPE followed by MS identified basic transcription factor-3 as being increased in dogs with lymphoma (Kycko *et al.* , 2018). Four autoantibodies, with potential application as biomarkers in both canine and human tumours were identified using a combination of 2D-PAGE and MS on the serum of dogs with mammary neoplasms (Zamani-Ahmadmahmudi, Nassiri and Rahbarghazi, 2014).

A variety of different bodily fluids and tissues have also been assessed in an attempt to discover potential protein biomarkers for dogs with cancer. Analysis of tears by 2D-PAGE and MALDI MS from dogs with a variety of neoplasms showed albumin and actin were reduced and a protein thought to be analogous to human lacryglobulin was increased (de Freitas Campos *et al.* , 2008). Canine mammary tumour tissue has been analysed using the combination of fractionation and identification of proteins. These analyses identified a difference in the protein expression of metastatic and non-metastatic tumours and when different stages

of malignant progression were compared. (Klopfleisch *et al.* , 2010; Klose *et al.* , 2011). Comparison of lymph node tissue from dogs with B-cell lymphoma, when compared to a control population identified both up- (macrophage capping protein) and down-regulated proteins (prolidase, triosephosphate isomerase, and glutathione S-transferase) with potential utility as biomarkers (McCaw *et al.* , 2007).

1.3.3.3.2. Two- Dimensional Difference Gel Electrophoresis

Two-dimensional difference gel electrophoresis (2D-DIGE) utilises the same principles as 2-dimensional electrophoresis in that the samples are separated in two dimensions. This technique minimises gel to gel variation by running more than one sample on the same gel with each sample being labelled by a separate dye. This allows differential expression of proteins to be identified, however identification of the protein in question still requires excision of a protein spot and MS (Matharoo-Ball *et al.* , 2008). MALDI-TOF was used following 2-dimensional difference gel electrophoresis to identify differences in the proteome of healthy dogs to those with a transected anterior cruciate ligament (Gharbi *et al.* .2013). Fetuin B and complement C3 were increased in diseased dogs and hyaluronan binding protein 2, inter- α -trypsin inhibitor H4, complement C1s and C4 were reduced.

Using 2D-DIGE, collagen XXVII peptide was increased in the serum of dogs with haemangiosarcoma. Collagen XXVII peptide also reduced after splenectomy and increased upon recurrence of the disease in two dogs (Kirby *et al.* , 2011). Proteomic expression profiling of canine osteosarcoma cells was performed using a combination of 2D-DIGE and MS to identify overexpression of MiR9 (Fenger *et al.* , 2016). When 2D-DIGE and MALDI-TOF MS were used to compare canine prostatic tumours to normal urothelium three overexpressed proteins, GRP78, GRP74 and keratin-7 were identified (LeRoy *et al.* , 2004).

The combination of 2D-DIGE and MS on tumour tissue identified a number of proteins which may be involved in the aetiology, pathogenesis or crucially the

biological behaviour of MCT (Schlieben *et al* . 2012). With MCT classified according to the two-tiered grading system, 13 proteins with significant differential expression were found. These proteins were grouped according to function and within groups of function, some proteins were up regulated while others were down regulated in HG MCT. Transferrin was down-regulated and stress response proteins such as HSPA9, PDIA3, TCP1A and TCP1E were up-regulated in HG MCT. Cell motility and metastatic proteins ANXA6, ACTR3 and WDR1 were also increased in HG MCT and the expression of two other proteins, ANXA2 and ACTB were decreased. When the differentially expressed proteins were further analysed, it was concluded that HG MCT have a higher resistance to cellular stress. HSPA9 has been identified as a therapeutic target as HSPA9 inhibitors are available as a therapeutic for human neoplasia.

1.3.3.3.3. One dimensional serum electrophoresis followed by polyacrylamide gel electrophoresis.

A novel technique employing 1D-SPE, followed by a 2D-PAGE and MS was used to evaluate the canine serum proteome (Atherton *et al* . 2013a). Individual protein bands were then excised from the gels and MS was used in combination with canine gene sequencing data to identify proteins. This did not require isoelectric focusing in the two-dimensional phase, which is technically demanding. The results were an improvement on previous work to characterise the serum proteome by Abate *et al.*, (2000) as the high resolution electrophoresis used previously was dependent on possessing antibodies to the proteins, meaning that only characterisation of known proteins could occur (Atherton *et al* . 2013a).

Thirty-two proteins were identified from the serum of two healthy dogs and as the proteins were identified in their specific globulin fraction, it was proposed that changes within the fraction on the 1D-SPE could possibly be used to identify diseased dogs. By comparing these results from the population of healthy dogs to that of three dogs with HG multicentric lymphoma (Atherton *et al* . 2013b), 10 proteins were identified that were not found in the normal samples. These

included clusterin and many established acute phase proteins, α 2 macroglobulin, inter- α -trypsin inhibitor, α anti-chymotrypsin and haptoglobin. Albumin levels were significantly reduced as was kininogen, possibly due to consumption as part of a pro-inflammatory process.

1.3.4. Neutrophil to lymphocyte ratio

As well as using serum proteins as biomarkers, circulating inflammatory cells have also been studied. The link between cancer and inflammation has long been established, but has received growing attention in recent years. Cancer promoting inflammation was added as an 'enabling characteristic' to the revised Hallmarks of Cancer (Hanahan and Weinberg, 2011). Inflammation may both play a role in the aetiology of cancer including canine MCTs (Govier, 2003) and be a consequence of it (Sideras and Kwekkeboom, 2014). Neutrophils are involved in angiogenesis and as tumour promoting inflammatory cells (Hanahan and Weinberg, 2011). Conversely, the tumour microenvironment may also be infiltrated by immunosuppressive cells such as T-regulator cells (Wang and Ke, 2011), other T and B lymphocytes, as well as neutrophils which can assume immunosuppressive properties (Hanahan and Weinberg, 2011). As a result of this immunosuppressive infiltration, cytotoxic lymphocyte numbers were reduced in comparison to normal populations in a study of a broad range of canine neoplasms (not including MCT) (Itoh *et al* ., 2009).

Taking advantage of these characteristics, the neutrophil to lymphocyte ratio (NLR) has been examined as a prognostic tool in many human cancers including haematological neoplasms (Sideras and Kwekkeboom, 2014). Over 60 studies in over 37,000 patients with various neoplasms were reported in a review, with a number of new studies since published and a further review highlighting the utility of the biomarker in breast cancer (Guthrie *et al* ., 2013; Ethier *et al* ., 2017).

Complete blood counts are recommended in the evaluation of MCTs which are in a location not amenable to surgery or if there is evidence of negative prognostic

factors (Welle *et al.* , 2008), Therefore use of this ratio as a biomarker would involve no further investigation for the dog or cost to its owner. A disadvantage of this ratio however is that both cell types can be influenced by a very wide range of unrelated processes (Sideras and Kwekkeboom, 2014) such as immunosuppressive and anti-inflammatory drugs and concurrent infections. The presence or absence of immune cells in the tumour micro-environment may also bear no relation to numbers within a peripheral sample (Sideras and Kwekkeboom, 2014).

To date and not including the published material from this thesis, there have been at least five studies examining the utility of the NLR in veterinary oncology (Macfarlane *et al.* , 2015; Mutz *et al.* . 2015; Skor *et al.* , 2017; Davies *et al.* , 2018; Fernandez and Chon, 2018). NLR was not identified as a significant prognostic variable in canine multicentric lymphoma (Mutz *et al.* , 2015). However, this is a diverse disease and this cohort was treated in a number of ways. It was suggested that further prospective evaluation of this biomarker may prove useful (Mutz *et al.* , 2015). Another ratio of haematological cells is prognostic for canine B-cell lymphoma, when treatment was standardised: the lymphocyte to monocyte ratio (Marconato *et al.* , 2015).

The NLR can be used to predict whether a soft tissue mass is a soft tissue sarcoma or benign soft tissue tumour. Above a threshold of 4.519, there is a high sensitivity of 95.5% and a specificity of 57.5% for identifying soft tissue sarcoma. Pre-treatment NLR may therefore allow for better treatment planning. NLR was not able to predict grade of soft tissue sarcomas in the same population (Macfarlane *et al.* , 2015).

On univariable analysis, NLR was found to be a significant prognostic variable for dogs with MCT with a variety of grades, treated with a variety of different modalities (Skor *et al.* , 2017). However, it was not an independent prognostic factor on multivariate analysis. NLR was a more useful tool than either of its constitutive parts (the absolute neutrophil and lymphocyte concentrations). However, retrospective analysis meant that treatment could not then be tailored

in response to differing NLRs. No comparison was made between NLR and grades of MCT to identify potential use in predicting grade and therefore to guide treatment decisions. The same study identified the neutrophil to eosinophil ratio as an independent prognostic variable, with a lower eosinophil count identifying shorter survival times.

1.4 Aims of this thesis

There are currently few objective measurements which can differentiate between LG and HG MCT without obtaining a sample for histology. However, biomarkers used in human and veterinary medicine might, if applied to MCTs, allow better differentiation. Identification of reliable biomarkers would allow more informed treatment decisions to be made and possibly help indicate the prognosis for each individual patient.

The aims of this thesis were:

1. To determine the potential use of NLR and AGR in predicting the biological grade of canine MCTs in dogs with gross tumour burden.
2. To identify novel serum biomarkers by searching for differences in the proteome of dogs with known HG and LG MCT and by comparing dogs with HG and LG MCT to healthy controls.

2 Methods

2.1 Case Selection

2.1.1. Dogs with Mast Cell Tumours

To assess suitability for inclusion, an electronic patient database was searched to identify all dogs presented to the University of Glasgow Small Animal Hospital between June 2008 and September 2015 with a definitive diagnosis of mast cell neoplasia. These dogs were presented for diagnosis and treatment of their disease as appropriate. Dogs were included into the study if they met the following criteria:

- A complete blood count and/or biochemical analysis were performed at the University of Glasgow Small Animal Hospital, whilst the dog had a gross MCT. This formed part of the clinical staging process.
- The dog was not receiving (or had received within the prior ten days) any medication with the potential to alter the serum proteome or numbers of circulating blood cells by any route of administration. This was considered to be any corticosteroid, anabolic steroid, phenobarbitone, ketoconazole, any chemotherapeutic agent, any tyrosine kinase inhibitor or any other immunomodulatory drug (oclacitinib, ciclosporin, azathioprine, mycophenolate, etc.).
- No co-morbidities were evident on clinical examination, clinical history or after analysis of clinical staging results which could influence either protein or leucocyte results. Examples include atopic dermatitis and protein losing enteropathy.
- No surgical procedures had been performed in the fourteen days prior to time of blood sampling.
- The diagnosis of a MCT was made histopathologically, allowing the tumour grade to be attained.

Excess blood, which was deemed surplus to requirements for haematological and biochemical profiling which formed part of the diagnostic investigation, was decanted into serum tubes (International Scientific Supplies Ltd, Bradford, UK). These samples were allowed to clot and then centrifuged at 7,000 rpm for three minutes (Minispin, Eppendorf, Hamburg, Germany). The serum was separated into another plain tube and stored at -80°C.

The following clinical factors were recorded from the medical record; age, sex, breed, tumor grade, location and size, presence of lymph node and distant metastasis, albumin and globulin concentrations, absolute neutrophil and lymphocyte count. Further staging evaluation (abdominal and/or thoracic imaging) was not a pre-requisite for inclusion but any staging procedures were documented. With regards to tumour size, the largest single dimension was used for single lesions, with the sum of the largest single dimension used in multiple MCTs (including lymph node metastasis). The WHO stage was also recorded where possible. The tumour location was divided into four groups, head and neck, extremity, other or multiple.

If histopathological grading was performed prior to the routine use of the two-tier, Patnaik grading system, tumours were re-graded to the Kiupel grading system by a single, board certified anatomic pathologist (Timothy Scase, Bridge Pathology Ltd.). In multiple MCTs, if any lesions were deemed HG, the dog was classified in the HG group.

2.1.2. Control Cases

Control samples were from six healthy adult dogs which were voluntarily offered by their owners for use as blood donors. These dogs had no known illnesses, had not received any medications in the preceding fourteen days and did not have any significant haematological or serum biochemical abnormalities. Serum was separated from these samples as for the study samples and stored under the same conditions.

The samples for both control and study dogs were taken after at least a ten hour fast. All procedures and the use of the samples were approved by the Research Ethics Committee of the College of Medicine, Veterinary and Life Sciences, University of Glasgow.

2.2 Neutrophil to Lymphocyte Ratio and Albumin to Globulin Ratio

Blood samples were decanted into potassium EDTA and lithium heparin tubes (International Scientific Supplies Ltd.). Albumin concentration was measured biochromatically using a bromocresol green dye-binding method and total protein concentration was measured using a biuret spectrophotometric assay in an automated analyser (Olympus AU640, Olympus, Tokyo, Japan). Globulin concentration was calculated as the difference between these values. An automated haematology analyser (Advia 2120 with veterinary package, Siemens, Munich, Germany) was used for haematological analysis. Results were verified using a manual differential from a wedge blood smear stained using May-Grünwald Giemsa Stain.

2.3 Proteomics

2.3.1. One dimensional Agarose Gel Electrophoresis

Protein electrophoresis was performed using a one-dimensional agarose gel (Hydragel Protein (E) K20, Sebia, Camberley, UK). Ten microlitres of serum from each dog was loaded onto the gel along with an animal-based control serum (Pathonorm H, Sero AS, Oslo, Norway). The loaded gel was then placed into a buffering solution of 0.092% W/V barbital, 0.515% W/V sodium barbital and 0.01% sodium azide solution (Tris-Barbital Buffer, Sebia) and electrophoresed for twenty minutes at a constant voltage of 100 mV. The gel was then fixed in acid alcohol for fifteen minutes before being dried at 37°C for at least sixteen hours. A stain (Amido Black, Sebia) was then applied for four minutes, and the gel was destained for a further four minutes (Destaining Solution, Sebia).

A digitally captured (UMAX PowerLock III flatbed scanner, UMAX UK Ltd, Milton Keynes, UK) colour image of this one dimensional gel was then used to perform densitometric analysis (ImageJ, National Institutes of Health, Bethesda, Maryland, USA) to quantify the percentage of the total protein present in each of six protein fractions, albumin, α -1, α -2, β -1, β -2 and γ .

The results were reviewed to establish a boundary of each fraction. The absolute values for each protein fraction were then calculated using the total protein result obtained previously from the same blood sample by the biuret method.

Six dogs (three with LG MCTs and three with HG MCTs) were then selected as clinically representative of their respective tumour grade along with three controls. 1D-SPE was repeated on these dogs as the first stage of a two dimensional electrophoresis. A further 10 μ l of serum was loaded into lanes one and three to seven and the gel was then electrophoresed and dried as described above. Lane one was then excised from the remainder of the gel and stained as described above. The six protein fractions were identified on this stained lane and it was then used as a guide to dissect the protein fractions from unstained lanes three to seven. The samples from lane three to seven were combined to ensure an adequate mass of protein was available with which to perform 2D-PAGE electrophoresis.

2.3.2. Polyacrylamide Gel Electrophoresis

The excised protein fractions from the 1D-SPE were then processed to extract the protein from the gel. Individual protein fractions were sectioned into small fragments and placed into a 0.5 ml Eppendorf tube (Fisher Scientific, Loughborough, UK). A buffer containing 43 μ l of Laemmli sample buffer (Biorad), 43 μ l of deionised water and 4.5 μ l mercaptoethanol (Sigma, Dermstadt, Germany) was added to the tube which was heated to 95°C for five minutes, then cooled and stored for 12 hours, before being heated to 95°C for a further five minutes. Ten microlitres of extracted protein was then used for 2D-PAGE (Criterion TGX Precast Gel, Bio-Rad, Hercules, California), with one lane used

for a protein ladder (Page Ruler Pre Stained Protein Ladder, Thermo Scientific, Waltham, USA) to facilitate comparison between gels.

The gels were electrophoresed at 300 V for fifteen minutes in a running buffer (Bio-Rad XT MOPS Running Buffer, BioRad Inc). Gels then were stained using a Coomassie Blue stain (Biosafe Coomassie Blue, BioRad Inc) for one hour with the gel placed on a rocking device. The gel was then rinsed in an acid alcohol solution (20% methanol and 7.5% acetic acid) prior to destaining for twelve hours in acid alcohol with rocking. The gel then was placed in distilled water for 24-48 hours.

Digital images of the polyacrylamide gels were then captured using a flatbed scanner (UMAX PowerLock III flatbed scanner) and protein bands within each lane were identified. Each identified band was then carefully excised from the gel. This was performed within a laminar flow hood using instruments which were rinsed in ethanol and distilled water after each band was excised. Each individual band was placed into a 0.5 ml Eppendorf tube and stored until required at -80°C.

2.3.3. Mass Spectrometry

Prior to MS, the protein within the gel pieces was digested using trypsin. Larger pieces were cut into smaller fragments and were washed in 500 µl of 100 mM ammonium bicarbonate (GE healthcare, Chicago, Illinois) for thirty minutes on a shaker. Gel pieces were then washed in 500 µl of 50% acetonitrile/100 mM ammonium bicarbonate (GE Healthcare) for thirty minutes with shaking. This second wash was then repeated. Fifty microlitres of acetonitrile was then added to shrink gel pieces for 10 minutes. The solvent was then removed and gel pieces were completely dried in a vacuum centrifuge. A sufficient amount of 20mg/ml of trypsin (Promega sequencing grade trypsin, Promega Ltd.) suspended in 25mM ammonium bicarbonate was then used to resuspend the pieces, which were then left for at least 12 hours at 37°C. Gel pieces were then pelleted and all liquid transferred to a ninety six well microplate (Thermo Scientific). Twenty

microlitres of 5% formic acid was added to the remaining gel piece and this was incubated for twenty minutes on a shaker. Forty microlitres of acetonitrile was then added to this, before a further twenty minutes of shaking. The gel was pelleted once more and the solution pooled with the previously removed solution in the ninety six well microplate. Samples were dried in a vacuum microcentrifuge and stored at -80°C until MS was performed. All the above steps were performed in a laminar flow hood.

For mass spectrometric analysis, stored tryptic peptides were solubilized in 0.1% formic acid and fractionated on a nanoflow Ultimate 3000 uHPLC system (Thermo Scientific) before being analysed by nanoelectrospray ionisation CaptiveSpray MS on an Amazon Speed ion trap MS/MS (Bruker Daltonics, Billerica, USA). Peptide separation was performed on a Pepmap100 C18 reversed phase column (Thermo Scientific), using a 4 - 40% v/v gradient (in 0.1% v/v formic acid, 80% acetonitrile) run over 23 min at a flow rate of 0.3 ml / min. This was followed by an increased gradient of 40-100 % v/v over 9 minutes. The column was washed for a further 5 minutes before returning to starting conditions. Next, a continuous duty cycle of survey MS scan was performed followed by up to ten tandem MS analyses of the most abundant peptides. These were selected using the most intense multiply charged ions with dynamic exclusion for 12 seconds.

MS data were processed using data analysis software (Bruker Daltonics) and the automated Matrix Science Mascot Daemon server (v2.5.0). Protein identifications were assigned using the Mascot search engine to interrogate Canifomia protein sequences in the National Centre for Biotechnology Information database (04 April 2015), allowing a mass tolerance of 0.4 Da for both MS and MS/MS analyses. Oxidation of methionine was considered and carbamidomethylation of cysteines was assumed. Mascot Molecular Weight Search (MOWSE) scores of 64 or greater indicated identity or extensive homology ($p < 0.005$). When proteins matched sequences from multiple species, only the species with the highest Mascot Molecular Weight Search score was included in the results unless a match with a

MOWSE score of over 64 within the species *Canine lupus familiaris* was detected and this was then included in preference.

2.4 Statistical Analyses

2.4.1. Neutrophil to Lymphocyte Ratio and Albumin to Globulin Ratio

All statistical analyses were performed using statistical software (IBM SPSS Statistics for Windows 22 & Minitab 16). Due to the number of cases and after visual inspection of Q-Q plots for independent variables, non-parametric analysis was used and data are presented as medians and ranges. Mann-Whitney U Tests were used to compare haematological and biochemical parameters, NLR and AGR between high and LG MCTs. Univariable and multivariable logistic regression analyses were used to evaluate the association of clinical factors and measured parameters to the MCT grade (high or low); factors with $p < 0.2$ in the univariable model were considered for inclusion in the multivariable analysis. This was assembled as a manual stepwise forward logistic regression with $p < 0.05$ considered significant. Goodness of fit was assessed using Hosmer-Lemeshow goodness of fit test. Listwise deletion was used for handling missing data. ROC curves were constructed in order to calculate the sensitivity and specificity of potential biomarkers for predicting histopathological grade.

2.4.2. One dimensional serum protein electrophoresis

All statistical analyses were performed using statistical software (IBM SPSS Statistics for Windows 22 & Minitab 16). Due to the number of cases and after visual inspection of Q-Q plots for independent variables, non-parametric analysis was used and data are presented as medians and ranges. Mann-Whitney U Test, Kruskal-Wallis and Fischer's exact tests were used where appropriate to compare protein concentrations between HG and LG MCTs and control groups.

3 Results

3.1 Use of the Neutrophil to Lymphocyte and Albumin to Globulin Ratios for Predicting Histopathological Grade of Canine Mast Cell Tumours

3.1.1. Introduction

The first aim of the thesis was to determine whether NLR and AGR were useful blood biomarkers for dogs bearing gross MCTs in terms of predicting tumour grade and outcome. Both NLR and AGR have been identified as biomarkers of interest in human neoplasia (Guthrie *et al.* , 2013; Aksoy *et al.* , 2016) and may therefore have utility in management of MCT. This could be in directing initial investigation, such as whether to perform full staging examination and also in guiding treatment decisions. MCT which are predicted to follow a rapidly progressive disease course are recommended to have different initial investigation and treatment (Blackwood *et al.* , 2012) and there is therefore a need for an objective, cost effective, minimally invasive, reliable and accurate parameter which can predict the biological behaviour of cutaneous mast cell tumours. In this chapter, NLR and AGR were compared between HG and LG MCT in a population of 61 dogs with naturally occurring MCT (62 tumours), using the complete blood count and biochemical results.

3.1.2. Neutrophil to Lymphocyte and Albumin to Globulin Ratios

Results presented in this section have previously been published elsewhere (MacFarlane *et al.* , 2016).

Sixty one dogs met the inclusion criteria outlined in the methods section 2.1 (Appendix 1). A male entire boxer had two LG MCTs, in different locations, temporally spaced by 4 years and was therefore included twice, so 62 sets of results were available: 14 (23%) HG and 48 (77%) LG MCTs.

Nineteen Labrador retrievers were included in the analyses (12 HG MCT, 7 LG MCT); 13 boxers all with LG MCT; 10 crossbred dogs, six of which had LG MCT; six golden retrievers all with LG MCT; three Staffordshire bull terriers (2 LG MCT, 1 HG MCT); two springer spaniels with LG MCT; there were one West Highland white terrier, beagle, miniature schnauzer, Border collie, Anatolian shepherd, Pyrenean mountain dog and vizsla with LG MCT and one Jack Russell terrier and Lhasa apso with HG MCT.

Thirteen male entire (9 LG MCT, 4 HG MCT), 23 male neutered (17 LG MCT, 6 HG MCT), six female entire (5 LG MCT, 1 HG MCT) and 20 female neutered dogs (17 LG MCT, three HG MCT) met inclusion criteria.

The median age for all dogs was seven years (range 1-14); for the LG group seven years (range 1-12) and the HG group 9.5 years (range 5-14). The distribution of ages in these groups is statistically significant ($p=0.004$).

Of the LG MCT: 21 were located on the extremities; 14 were located on the head or neck; eight dogs had multiple LG MCT and five had tumours in other locations. Of the HG tumours: four each were present on the extremities and on the head or neck; one dog had multiple MCT, of which one was HG and five had MCT in other locations.

Three LG MCT cases did not have available longest dimension data and this was therefore available for 59 cases. Overall, the median MCT size was 25mm. The median size of the LG MCT group was 21mm with a range 6-80mm and there was a median of 36mm and range of 12-140mm for HG MCT. There is a significant difference in tumour size between groups ($p=0.026$).

No major systemic effects of the MCT were reported in either group. Four dogs from the LG MCT (8%) and HG MCT groups (29%) had local clinical signs. These local signs were: pruritus in three cases, erythema of the lesion in two cases, ulceration of the MCT in two cases or local oedema and swelling in one case.

Six dogs with LG MCT had palpably enlarged local lymph nodes. Five of these were confirmed as metastatic histologically and one was classed as free of metastasis after cytological examination. Thirteen LG cases had palpably normal lymph nodes, one was found to be metastatic histopathologically, six were found to be non-metastatic by cytological exam and two by histopathological exam. Seven dogs in the HG MCT group were reported as having enlarged lymph nodes. Three were confirmed as metastatic histopathologically and three were confirmed cytologically. One HG MCT with an enlarged local lymph node was not found to have any mast cells on cytological examination of the lymph nodes. Four local lymph nodes in the HG group were reported as normal sized and not examined. The size of the local lymph node was not recorded for 28 LG MCTs and three HG MCT and cytology nor histopathology were performed. Therefore, in six each of the HG and LG MCT groups, there was evidence of lymph node metastasis.

All MCT cases had staging including at least abdominal ultrasound and thoracic radiographs (two or three views) on day of blood sample collection, facilitated with sedation. If either the liver or spleen was considered to be significantly abnormal in appearance, an FNA and cytological analysis was performed. Results are detailed in Table 3-1. The liver was increased in size and increased in echogenicity in two cases each. Focal lesions and a heterogenous parenchyma were identified in one case each. A heterogeneous splenic parenchyma was identified in five cases and focal splenic lesions in two cases. There was both a heterogenous parenchyma and an increase in size of the spleen in a further two cases.

FNA of the spleen was performed in 23 cases and hepatic FNA in nine dogs (Table 3-1). No mast cells were seen in five of the liver samples, with occasional mast cells in four. In nine of the splenic samples, there were no mast cells and there were occasional, well differentiated mast cells in the remainder. The occasional mast cells were not considered to represent metastatic disease in any sample. There was no concerning abnormality identified on any thoracic radiograph,

therefore no distant metastatic disease was identified in any case at time of inclusion.

Grade	Number of cases	Hepatic ultrasound abnormalities	Hepatic FNA	Metastatic disease suspected	Splenic ultrasound abnormalities	Splenic FNA	Metastatic Disease Suspected
<u>Low</u>	48	3	6	0	6	15	0
<u>High</u>	14	3	3	0	3	8	0
<u>Total</u>	62	6	9	0	9	23	0

Table 3-1: Hepatic and splenic ultrasonographic abnormalities in dogs with gross mast cell neoplasia. FNA= fine needle aspirate

The majority of tumours from dogs in LG and HG groups were considered WHO stage I MCTs. No dogs had stage IV MCT. A full breakdown of tumour stages is shown in Table 3-2 and shown for each individual case in Appendix 1.

<div> <div>MCT Grade</div> <div>WHO Stage</div> </div>	Low	High	Total
<u>I</u>	34	7	41
<u>II</u>	6	6	12
<u>III</u>	8	1	9
<u>IV</u>	0	0	0
<u>Total</u>	48	14	62

Table 3-2: World Health Organisation stage (Owen, 1980) distribution amongst different grades of cutaneous mast cell tumour being analysed for biomarker identification WHO= World Health Organisation, MCT= mast cell tumour.

Complete blood count results were available for all 62 cases. When the individual components of the NLR were considered, only lymphocyte concentration was significantly different between MCT grades, being lower in the HG MCT group ($p=0.001$). NLR was significantly higher in dogs with a HG MCTs (Figure 3-1) ($p<0.001$).

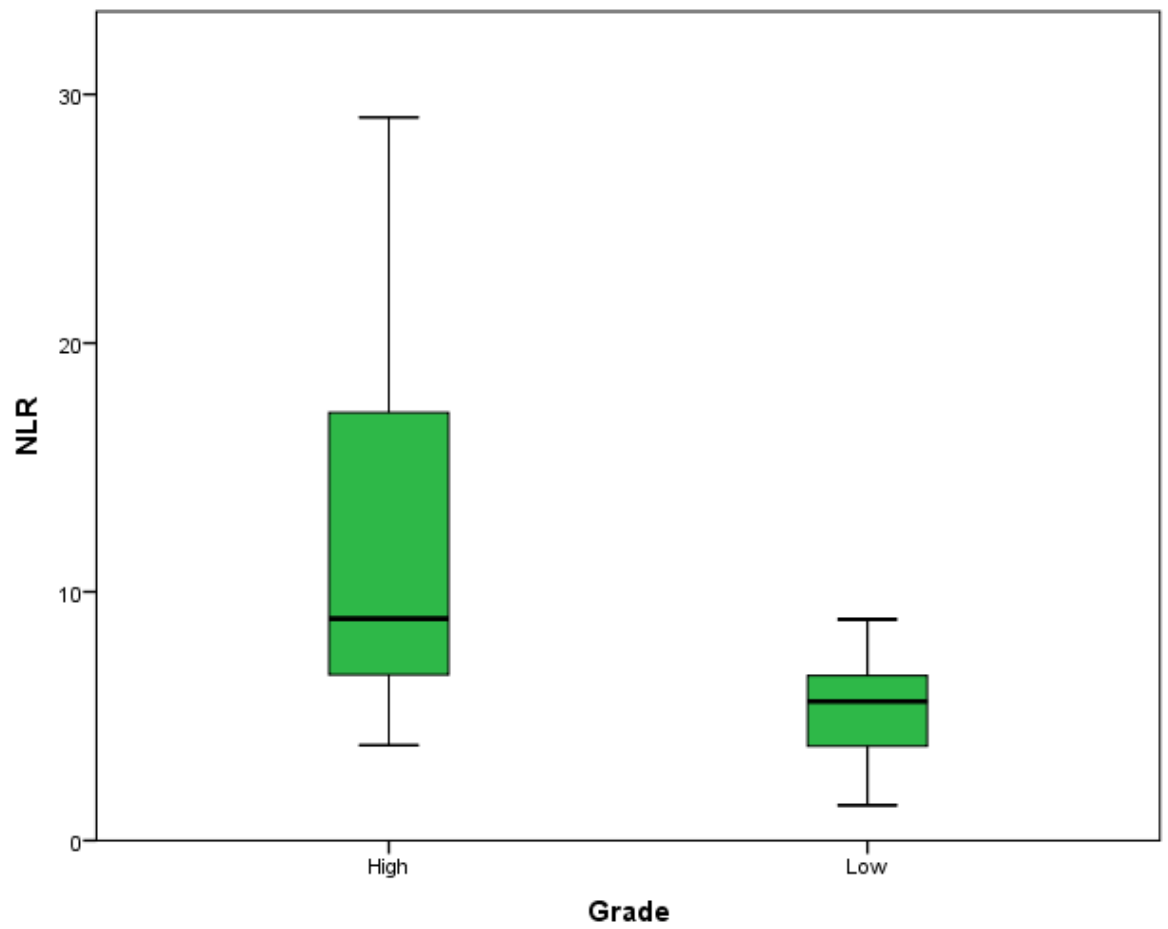


Figure 3-1: Box and whisker plot showing the neutrophil to lymphocyte ratio in grades of cutaneous mast cell tumour. NLR= neutrophil to lymphocyte ratio.

Biochemical results were not available for one dog with a HG MCT. No significant difference was found when comparing either the individual components of the AGR or the AGR itself between LG and HG MCTs. Statistical comparison of absolute counts, NLR and AGR between tumour grades are shown in Table 3-3.

Grade Test	All	High grade	Low Grade	p-value
<u>Albumin concentration (g/l)</u>	32 (21-36)	29 (21-36)	32 (27-36)	0.413
<u>Globulin concentration (g/l)</u>	34 (27-45)	34 (27-45)	33.5 (28-43)	0.318
<u>AGR</u>	0.94 (0.6-1.25)	0.86 (0.6-1.09)	0.94 (0.71-1.25)	0.061
<u>Absolute neutrophil count</u> <u>(x10⁹/l)</u>	6.66 (3.2-15.18)	8.32 (3.62-15.18)	6.48 (3.2-12.01)	0.053
<u>Absolute lymphocyte count</u> <u>(x10⁹/l)</u>	1.16 (0.21-3.25)	0.85 (0.21-1.34)	1.22 (0.65-3.25)	0.001*
<u>NLR</u>	6.00 (1.42-29.07)	9.15 (3.83-29.07)	5.59 (1.42-8.89)	0.000*

Table 3-3 Median values and (ranges) for biomarkers of interest of dogs with high and low grade mast cell tumours. AGR=albumin to globulin ratio, NLR=neutrophil to lymphocyte ratio, * denotes a significant result

Univariate analysis was then performed, with the aim of identifying factors which could be built into a multivariable model to identify factors which could predict MCT grade. Fourteen variables were initially assessed for their association with MCT grade (Table 3-4). Twelve parameters (NLR, absolute lymphocyte count, age, tumour size, absolute neutrophil count, presence of lymph node metastasis, AGR, WHO tumour stage, presence of clinical signs, albumin concentration, globulin concentration and tumour location) were found to have a P value under 0.2 and were then included in the multivariable model. Sex and neutered status had P values above 0.2 and were not included.

Factor	Odds Ratio (95% Confidence Interval)	P value
Age	1.47 (1.13-1.94)	0.001*
Neuter Status		0.643
Neutered relative to Entire	1.34 (0.38-4.75)	
Sex		0.242
Female relative to male	0.47 (0.13-1.71)	
Tumour Location		0.169*
Body relative to Head	3.5 (0.66-18.49)	
Extremity relative to Head	0.67 (0.14-3.11)	
Multiple relative to Head	0.44 (0.04-4.62)	
Limb relative to Body	0.19 (0.03-0.98)	
Multiple relative to Body	0.12 (0.01-1.41)	
Multiple relative to Limb	0.65 (0.06-6.80)	
Tumour Size	1.04 (1.01-1.07)	0.006*
Lymph Node Metastasis	5.25 (1.35-20.47)	0.018*
WHO stage		0.054*
II relative to I	4.86 (1.21-19.57)	
III relative to I	0.61 (0.07-5.66)	
III relative to II	0.12 (0.01- 1.33)	
Clinical Signs	4.4 (0.94-20.66)	0.065*
Neutrophil Count	1.39 (1.04-1.84)	0.015*
Lymphocyte Count	0.02 (0.00-0.29)	0.000*
NLR	1.83 (1.21-2.76)	0.000*
Albumin Concentration	0.83 (0.67-1.04)	0.097*
Globulin Concentration	1.11 (0.95-1.30)	0.163*
AGR	0.04 (0.00-0.70)	0.027*

Table 3-4: Showing the univariate logistic regression identifying factors significant in predicting mast cell tumour grade. A factor with a value of $p < 0.20$ was considered for a multivariate stepwise logistic regression analysis. * denotes $p < 0.2$ and factor part of multivariable analysis. WHO= World Health Organisation. NLR= neutrophil to lymphocyte ratio. AGR= albumin to globulin ratio.

NLR and age remained within the final multivariable model. For each extra year of age the likelihood of HG MCT increased by 1.67 times (95% confidence interval: 1.08-2.59; p-value =0.005). Each unit increase in NLR was associated with an increase in the likelihood of HG MCT of 2.03 times (95% confidence interval- 1.24- 3.35; p-value < 0.001) (Table 3-5). To assess whether the data conflicted with assumptions made by the model, the Hosmer-Lemeshow goodness of fit test was performed. This provided no evidence of a lack of fit (p=0.128).

Factor	Odds Ratio (95% Confidence Interval)	P value
NLR	2.03 (1.24-3.35)	<0.001
Age	1.67 (1.08-2.59)	0.005

Table 3-5: Stepwise multivariate logistic regression identifying factors which can predict mast cell tumour grade. All factors with a $p < 0.2$ on univariate analysis were considered. Hosmer Lemeshow goodness of fit test- $p = 0.128$. NLR= neutrophil to lymphocyte ratio.

The identification of a predictive cut-off value for each biomarker was considered to be clinically useful. In order to facilitate this, ROC curve analysis indicated that the area under the curve was 0.84 for NLR (Figure 3-2). Analysing the data produced by this test, a NLR threshold value of 5.67 gave a sensitivity of 85.7% and a specificity of 54.2% for predicting HG disease, correctly classifying 61% of MCTs. When the same test was performed using the AGR, the area under the curve for was not deemed high enough to be a useful discriminatory test (Figure 3-3).

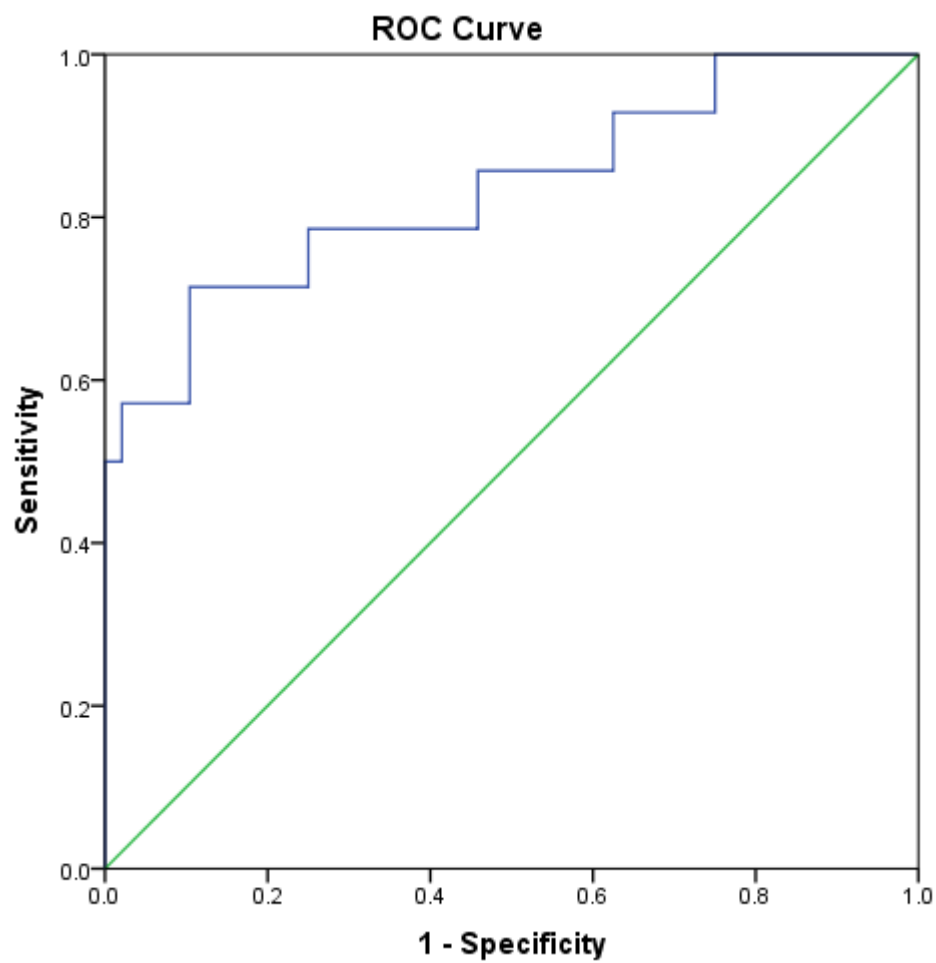


Figure 3-2: Receiver operator characteristics curve for neutrophil to lymphocyte ratio, evaluating ability to predict mast cell tumour grade. Area under the curve= 0.84 (confidence interval 0.70-0.97). ROC= receiver operating characteristic.

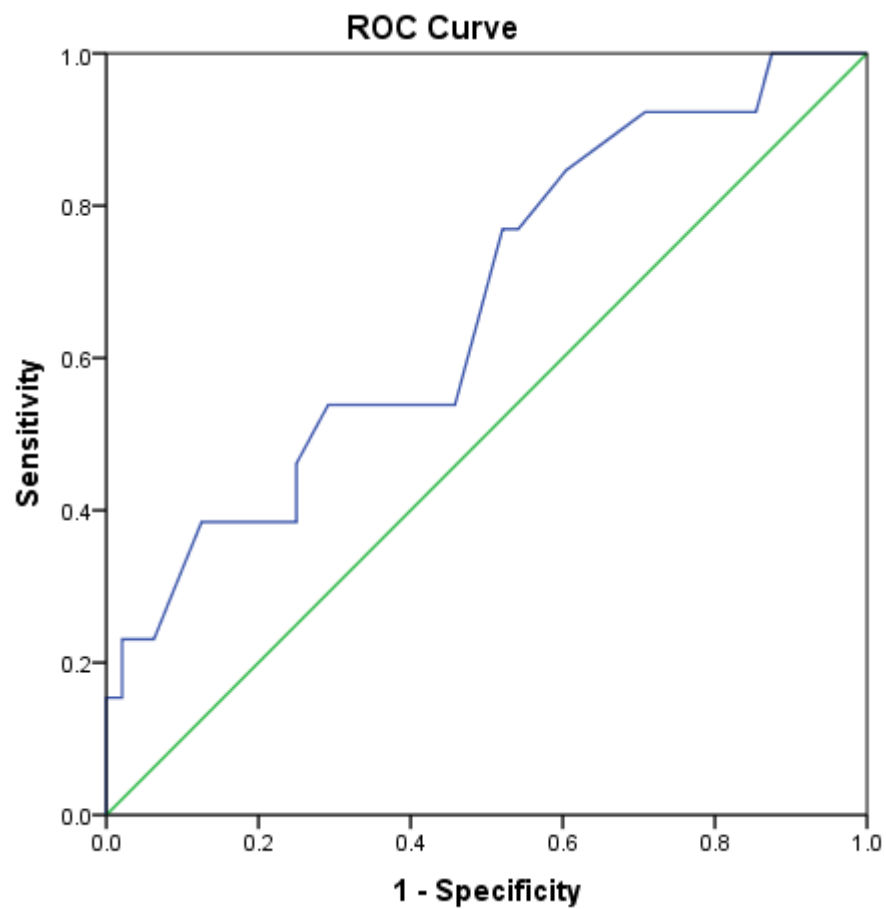


Figure 3-3: Receiver operating characteristics curve for albumin to globulin ratio evaluating ability to predict mast cell tumour grade. Area under the curve= 0.67 (confidence interval 0.50-0.84). ROC= receiver operating characteristic

3.2 Comparing the Serum Proteome of High and Low Grade Canine Cutaneous Mast Cell Tumours

3.2.1. Introduction

Rather than just focussing on biomarkers already identified in the literature, the second aim of the thesis was to identify novel serum biomarkers to help separate LG from HG MCT and to separate MCT from controls.

The differing molecular mechanisms occurring within LG and HG MCTs induce changes in the transcription and translation of proteins involved in a wide range of functions, such as cell motility, proliferation, metastasis and stress responses within the tumour (Schlieben *et al.* . 2012). MCT result in changes to serum acute phase proteins such as C-reactive protein and α -1 acid glycoprotein which are increased above reference ranges (Chase *et al.* ., 2012). Despite this, little is known regarding more detailed changes to the serum electrophoretogram and the serum proteome, especially any differences between LG and HG MCT.

Differences between globulin fractions have been identified in canine lymphoma using 1D-SPE (Atherton *et al.* ., 2013b). In order to assess whether similar differences were present in MCT, the first part of this results section shows how 1D-SPE was performed on 20 dogs with MCT to create electrophoretograms on the basis of densitometric scanning to analyse concentrations of protein fractions. In order to assess which specific proteins contributed to any differences identified using the 1D-SPE technique, and therefore to identify novel biomarkers of MCT and to compare serum changes to those within MCT tissue (Schlieben *et al.* ., 2012) a more in depth method of analysis was required. Based on its successful application in dogs with lymphoma, a previously described (Atherton *et al.* ., 2013b) method of further protein separation using 2D-PAGE and MS was then used on six of these tumour bearing dogs and the results are presented in the second part of this section.

1D-SPE was repeated to allow a primary method of separation in preparation for MS. 2D-PAGE without isoelectric focusing was then used to allow sufficient

separation to allow MS and identification of individual proteins within the serum samples of dogs with MCT. The ultimate aim was identification of both quantitative differences in serum protein fractions between LG and HG MCT and control cases as well as the presence or absence of specific serum proteins, which could identify discriminatory biomarkers for HG MCTs.

3.2.2. Case Selection

Twenty dogs with MCT which met inclusion criteria listed in the methods section 2.1.1 were selected retrospectively on the basis of having the expected clinical outcome predicted for their tumour grade (e.g. no recurrence or metastasis for LG MCT and vice versa for HG MCT) and sufficient serum left to allow analysis. This included 12 LG MCT and eight HG MCT. In the LG group, there were three each of boxers and crossbreeds, two Labrador Retrievers and one each of golden retriever, cocker spaniel, beagle and Border collie. There were seven male entire, three female neutered and two male neutered dogs. Median age was eight years with a range of three to eleven years. Median total protein was 63.5 g/l (range 62-72 g/l).

Of the eight dogs with a HG MCT which were included, there were four crossbreed dogs, two Labrador retrievers and one each of Jack Russell terrier and Staffordshire bull terrier. There were three male entire and female neutered dogs and two male neutered dogs. Median age was 8.5 years with a range of five to 14 years. Median total protein was 66.5 g/l (range 57-78 g/l).

Due to the requirements for each laboratory to establish their own reference ranges for 1D-SPE (Tappin *et al* ., 2011), published ranges could not be used to assess differences in the serum electrophoretogram between control and LG and HG MCT groups. Therefore, the serum from six control dogs was used to establish normal values for each protein fraction. The demographics of the three groups of dogs - control, LG MCT and HG-MCT are shown in Table 3-6. Of the six control dogs, there were three flat-coated retrievers, one Labrador retriever, one Leonberger and one greyhound. Four were male neutered and two female

neutered dogs. Median age was 3.5 years with a range of two to five years. Median total protein concentrations for this group was 63g/l (range 61-70g/l).

	#	Age	Sex	Breed	Total Protein	Biochemical abnormalities	Haematological abnormalities	NLR	MCT size	MCT location	Clinical signs	MCT stage 79
<u>Control</u>	6	3.5 (2-5)	4 FN 2 MN	Flat-coated retriever (n=3) greyhound, Leonberger (both n=1)	63 (61-70)	None	Increased Hb (n=1)	4.63 (3.65-5.10)	N/A	N/A	N/A	N/A
<u>Low Grade</u>	12	8 (3-11)	8 ME 3 FN 2 MN	Boxer dog, crossbreed (n=3), Labrador retriever (n=2), golden retriever, cocker spaniel, beagle, Border collie (n=1)	63.5 (62-72)	Hypophosphatemia (n=11), Increased AST (n=3), hyperglycaemia, hypoalbuminemia, increased ALT (n=1)	Lymphopenia (n=4), increased MCH (n=2). Increased Hb, decreased Hb, increased MCV, leucocytosis (n=1)	6.54 (2.91-8.89)	18 (9-40)	Extremity (n=10), head/neck, other (n=1)	Pruritus (n=1)	Stage I (10) Stage II (2)
<u>High Grade</u>	8	8.5 (5-14)	3 FN 3 ME 2 MN	Crossbreed (n=4), Labrador retriever (n=2), Jack Russell terrier, Staffordshire bull terrier (n=1).	66.5 (57-78)	Hyperglycaemia (n=5), increased AST, hypophosphatemia (n=3), hypoalbuminemia (n=2), increased ALT, hypocalcaemia (n=1)	Lymphopenia (n=6), leucocytosis (n=3), neutrophilia, thrombocytosis (n=1)	9.83 (6.20-28.6)	36 (12-140)	Other (n=4), extremity (n=2), head/neck, multiple (n=1)	Pruritus (n=1)	Stage I (3) Stage II (4) Stage III (1)

Table 3-6: Demographics and tumour specifics in groups of healthy control, low grade and high grade mast cell tumour cases. Age in years, total protein in g/l, MCT size in millimetres, MCT Stage refers to the World Health Organisation staging system (Owen, 1980). ALT= alanine aminotransferase, AST= aspartate aminotransferase, FE= female entire, FN= female neutered, Hb= haemoglobin, MCH= mean corpuscular haemoglobin, MCT= mast cell tumour, ME= male entire, MN= male neutered. Number values are shown as median values and (range).

Dog age differed significantly different between the control and LG ($p=0.03$) and control and HG MCT groups ($p=0.01$), but not between HG and LG MCT groups ($p=0.473$). Tumour size was significantly different between HG and LG MCT groups ($p=0.012$).

Although dogs with MCT had some haematological and biochemical abnormalities, none were considered clinically significant. An increased haemoglobin concentration was identified in one control dog, which may have represented a normal value for the breed (greyhound).

NLR was significantly different between HG and LG MCT groups ($p=0.007$), HG and control groups ($p=0.011$) and LG and control groups ($p=0.04$).

However, no significant difference in serum total protein concentration was found between the three groups ($p=0.419$). No dogs had either an increased or decreased total protein concentration (range 50-78 g/l).

3.2.3. One-dimensional serum protein electrophoresis

Median values of absolute concentrations within the six protein fractions are shown in Table 3-7. Concentration of α -2 proteins were significantly increased in HG compared to LG MCT ($p=0.031$). Concentration of α -2 ($p=0.043$) and β -1 ($p=0.04$) fractions were increased in the HG MCT group compared to controls. Concentration of β -2 proteins was significantly decreased in HG compared to controls ($p=0.02$).

<u>Fraction</u>	Control median (range) g/l	Low Grade MCT median (range) g/l	High Grade MCT median (range) g/l
<u>Albumin</u>	48.2 (34.9-51.8)	43.0 (36.4-47.32)*	42.8 (34.6-45.32)*
<u>α-1</u>	4.0 (3.3-5.4)	4.4 (3.9-5.4)	5.0 (3.4-6.1)
<u>α-2</u>	17.6 (12.4-19.1)	17.6 (14.4-21.3)	19.9 (17.8-23.4)*†
<u>β-1</u>	6.8 (5.5-6.8)	9.4 (7.0-13.6)*	11.3 (6.1-18.0)*
<u>β-2</u>	13.4 (13.1-15.1)	13.3 (8.8-21.1)	11.1 (10.2-16.7)*
<u>γ</u>	10.9 (7.3-12.7)	9.8 (8.4-14.0)	8.8 (6.7-13.2)

Table 3-7: Relative serum protein fractions derived from one-dimensional serum protein electrophoresis in healthy dogs and those with low and high grade mast cell tumours. MCT=mast cell tumour. * denotes result significantly different from control group. †denotes result significantly different from low grade MCT group.

3.2.4. Two-dimensional polyacrylamide gel electrophoresis

After 1D-SPE, three each of control, LG MCT and HG MCT serum samples were selected for further protein separation and MS, as best representing the median relative protein concentrations for each respective group. Details of each dog are presented in Table 3-8. One-dimensional gels were repeated for the nine dogs, followed by PAGE as outlined in the methods section 2.3.2.

For each group of three samples (control, LG MCT, HG MCT), the stained PAGE gels were compared by visual inspection to establish the pattern of bands which could be expected as representative of the group.

Dog	Group	Age (years)	Sex	Breed	Tumour Size	Primary Tumour Location	NLR	Staging	Staging Abnormalities	WHO Stage	Treatment	Outcome
1	Control	5	FN	Leonberger			5.10					
2	Control	2	MN	Flat-coated retriever			3.65					
3	Control	3	MN	Greyhound			3.82					
4	LG	7	ME	Labrador retriever	9	Head/neck	4.53	CXR, AUS, cLN	None	I	Excision MCT	Alive 249 days, no recurrence
5	LG	11	FN	Crossbreed	22	Extremity	8	CXR, AUS, cLN	Metastatic superficial cervical LN	II	Excision MCT & LN	Alive 566 days, no recurrence
6	LG	11	FN	Crossbreed	15	Extremity	5.57	CXR, AUS.	None	I	Excision MCT	Alive 389 days, no recurrence
7	HG	9	FN	Crossbreed	25	Extremity	8.7	CXR, cAUS, hLN.	None	I	Excision MCT VBL/Pred	Dead 1030 days, recurrent disease
8	HG	14	FN	Crossbreed	60	Other	28.63	CXR, cAUS, hLN.	Metastatic axillary LN	II	Excision MCT & LN. VBL/Pred	Dead 310 days, recurrent disease
9	HG	8	MN	Labrador retriever	32	Other	10.5	CXR, cAUS,	None	I	Pred	Dead 30 days, progressive disease

Table 3-8: Case details of dogs selected for two-dimensional serum protein electrophoresis and mass spectrometry. AUS= abdominal ultrasound, cAUS=abdominal ultrasound with FNA and cytology of liver and spleen, CXR= thoracic radiographs, HG= high grade mast cell tumour, FE=female entire, FN=female neutered, LG= low grade mast cell tumour, LN= lymph node, cLN=FNA and cytology of local lymph node, hLN histopathology of local lymph node, MCT=mast cell tumour, ME=male entire, mm=millimetres, MN=male neutered, Pred= prednisolone, VBL= vinblastine. Staging using World Health Organisation system (Owen 1980).

3.2.4.1. *Controls*

Ten protein bands were identified in dog 1, with 15 bands identified from dogs 2 and 3. A protein band was identified in 21 unique positions, with seven of these bands identified in all gels, five in two gels and nine only in one gel (Figure 3-4). To establish which proteins migrated in each band, selected bands were excised for MS, focussing on those found in more than one gel and taking the band from whichever gel had the darkest staining band. Due to financial constraints, it was not possible to process all bands seen or to compare proteins present within bands on the same position in two or more gels. Albumin bands were not submitted as these proteins were less likely to yield potential biomarkers. Eleven bands were excised and MS was performed as described in the methods section 2.3.3. This analysis identified 22 proteins (Table 3-9). Of these proteins, albumin was identified in both the α -1 and β -1 fractions. There were five forms of immunoglobulin within the β and γ fractions and three complement related proteins from the β -1 fraction. Two histone proteins were present within the β -2 fraction. Of the 11 other proteins identified, one was as yet uncharacterised, three are involved in iron transport and haptoglobin, an acute phase protein, was identified in the α -2 fraction.

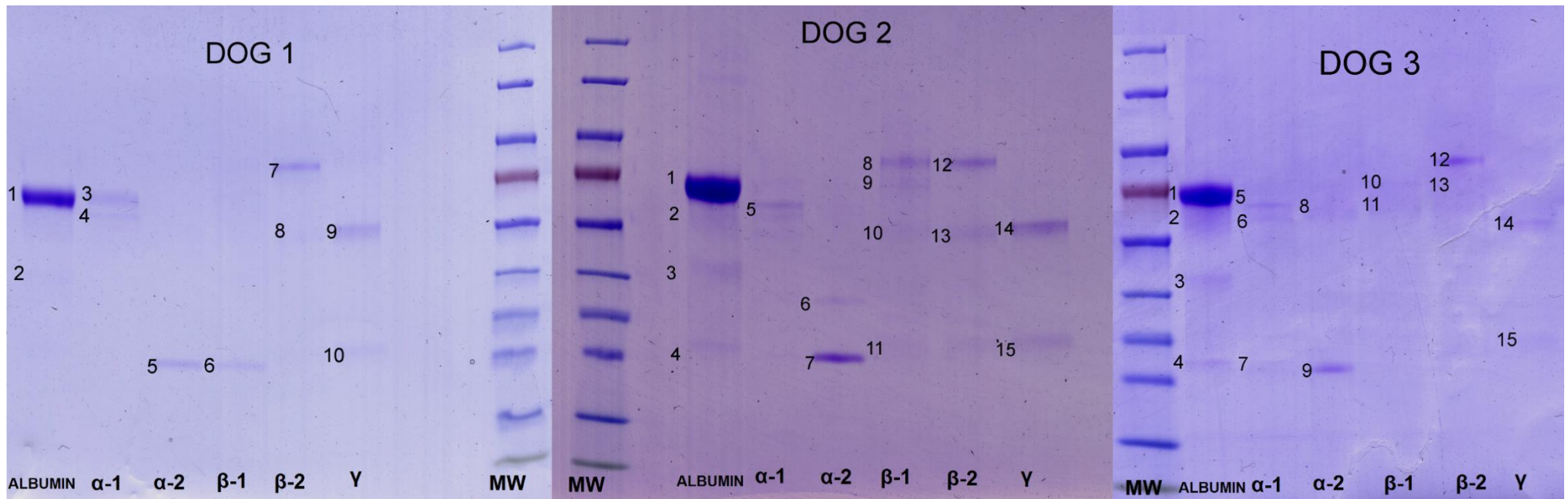


Figure 3-4: Image of two-dimensional serum protein electrophoresis gels from three control dogs. Each band and protein fraction has been labelled. Bands present in more than one gel were then selected to perform mass spectrometry. Dog 1 was a 5 year old female neutered Leonberger, dog 2 a 2 year old, male neutered flat-coated retriever and dog 3, a 3 year old, male neutered greyhound. Excised bands submitted for mass spectrometer analysis: dog 1- bands 3,7 and 8; dog 2- bands 6,7,8,9,11,14 and 15.

Protein Fraction	Protein identified by mass spectrometry	Dog(s)	Band(s)	MOWSE score(s)
α -1	albumin	1	3,	1513,
β -1		2	8, 9	69, 107
α -1	<i>alpha-1B-glycoprotein</i>	1	3	102
α -1	haemoglobin subunit alpha	1	3	112
α -1	<i>kininogen-1 isoformX2</i>	1	3	94
α -2	<i>apolipoprotein A-I</i>	2	7	1511
β -1			11	323
γ			15	92
α -2	<i>glial fibrillary acidic protein isoformX2</i>	2	7	132
β -2		1	7	83
α -2	haptoglobin beta chain	2	6	65
β -1	<i>C4b-binding protein alpha chain isoform X1</i>	2	8	119
β -1	<i>complement C3</i>	1, 2	7, 8, 9, 11	225, 86, 986, 102,
β -1	<i>complement C4-A</i>	2	8	71
β -1	<i>haemopexin</i>	2	9	153
β -1	<i>immunoglobulin lambda-like polypeptide 5-like</i>	2	11	143
γ			15	338
β -1	<i>serotransferrin isoform 1</i>	1	7	1527
β -2		2	8, 9	1385, 186
β -2	<i>14-3-3 protein sigma</i>	1	8	78
β -2	<i>histone H2B type 1-N-like</i>	1	8	74
β -2	<i>histone H4-like sequences</i>	1	8	186
β -2	immunoglobulin gamma heavy chains	1, 2	8 14	149, 146 306, 190
β -2	immunoglobulin heavy chains	1	7 8	83, 116
β -2	immunoglobulin heavy chain variable region	2	14	122
β -2	junction plakoglobin	1	8	95
β -2	plakophilin-1	1	8	120
β -2	<i>uncharacterized protein</i>	1	8	225

Table 3-9: Proteins identified by mass spectrometry from the serum of healthy control dogs after separation using a method of two-dimensional electrophoresis. Italic text denotes that proteins predicted from known genomic sequences. MOWSE= Mascot molecular weight search.

3.2.4.2. *Low Grade Mast Cell Tumours*

Nineteen of the 21 bands identified in the control population were also present on comparison of the three gels from the LG MCT dogs (Figure 3-5). However, 22 unique positions were additionally identified in LG MCT cases. Seven bands were identified in all gels, six in two gels and nine in one gel. As for controls, globulin bands for MS were excised from whichever gel the band stained most strongly, with 11 excised in total, identifying 17 proteins (Table 3-10). Of these 17 proteins, five were immunoglobulins, albumin was identified and three were proteins involved in oxygen transport. Complement C3 and haptoglobin were also found, within the β and α -2 fractions respectively.

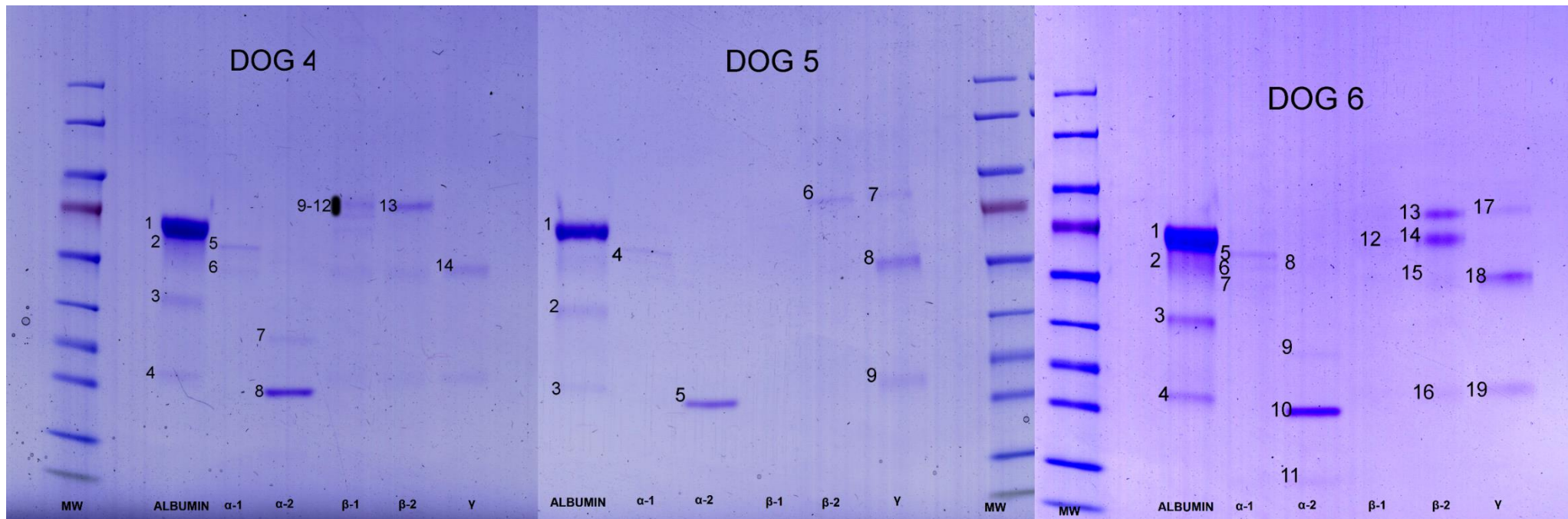


Figure 3-5: Image of two-dimensional serum protein electrophoresis gels from three dogs with low grade mast cell tumours. Each band and protein fraction has been labelled. Bands present in more than one gel were then selected to perform mass spectrometry. Dog 4, a 7 year old, male entire golden retriever with a 9mm mast cell tumour of the head/neck region; dog 5, an 11 year old female neutered crossbreed with a 22mm MCT on an extremity; dog 6, another 11 year old crossbreed with a 15mm MCT on an extremity. All were treated with surgery with no reported recurrence for a minimum of 249 days. Excised bands submitted for mass spectrometer analysis: dog 3- band 7; dog 5- band 4; dog 6- bands 10,12,13,14,15,17,18 and 19

Protein Fraction	Protein identified by mass spectrometry	Dog	Band (s)	MOWSE Score(s)
α -1	serpin peptidase inhibitor, clade A (alpha-1-antiproteinase) ^a	5	4	391
α -1	<i>kininogen-1 isoformX2</i>	5	4	218
α -1	<i>alpha-1 antitrypsin</i>	5	4	105
α -2	apolipoprotein A-I	6	10	1877
α -2	haptoglobin	4	7	354
β -1	Complement C3	6	12, 14	656, 105
β -2				
β -1	haemopexin	6	12	180
β -2	albumin	6	13, 17	274, 220
γ				
β -2	Immunoglobulin heavy chain variable region	6	13 18	69, 69 98
β -2	immunoglobulin gamma heavy chains	6	15 18	178, 113, 112 305, 282, 253, 96
γ				
β -2, γ	serotransferrin isoform 1	6	14, 15 17	473, 75 1076
β -2	Immunoglobulin kappa chain variable region ^a	6	16 19	83 81
γ				
β -2	<i>immunoglobulin lambda-like polypeptide 5-like</i>	6	16 17, 19	195 78, 274
γ	beta-2-glycoprotein 1 precursor ^a	6	18	85
γ	haemoglobin subunit β	6	17, 19	358, 101
γ	hypothetical proteins	6	19	94, 94
γ	immunoglobulin lambda light chain variable region ^a	6	19	75

Table 3-10: Proteins identified from the serum of dogs with low grade cutaneous mast cell tumours after separation using a method of two-dimensional electrophoresis. Italic text denotes that proteins predicted from known genomic sequences. ^a denotes proteins not found in the serum of healthy control dogs. MOWSE= Mascot molecular weight search.

3.2.4.3. *High Grade Mast Cell Tumours*

Fifty-three bands were identified from HG-MCT cases of which thirty were unique. Eleven of these unique bands were identified in all gels, one band in two gels and eighteen on one gel (Figure 3-6). Gels for dogs 7 and 8 had a similar number of bands to control dogs but dog 9 contained many of these same bands but also all 18 of the unique bands in the HG cohort. These were particularly in α_2 , and γ fractions. This dog had the shortest survival of the HG MCT. Fifteen globulin bands were excised and 30 proteins were identified (Table 3-11). Due to the number of unique protein bands seen, the majority of these were from dog 9. Of these proteins, six were immunoglobulins, haptoglobin and albumin were also identified, as were three complement related proteins. There were several proteins of interest, which have previously been found to be implicated in cancer pathogenesis. These were inter- α -trypsin inhibitor heavy chain H1 isoform X2, leucine-rich α_2 -glycoprotein, plasma protease C1 inhibitor, pregnancy zone protein, α_2 -macroglobulin isoform X2 and α_2 heat-shock glycoprotein (Starcevic, Jelic-Ivanovic and Kalimanovska, 1991; Bourguignon *et al.* , 1999; Jae *et al.* , 2009; Andersen *et al.* , 2010; Atherton *et al.* , 2013b; Adeola *et al.* , 2015).

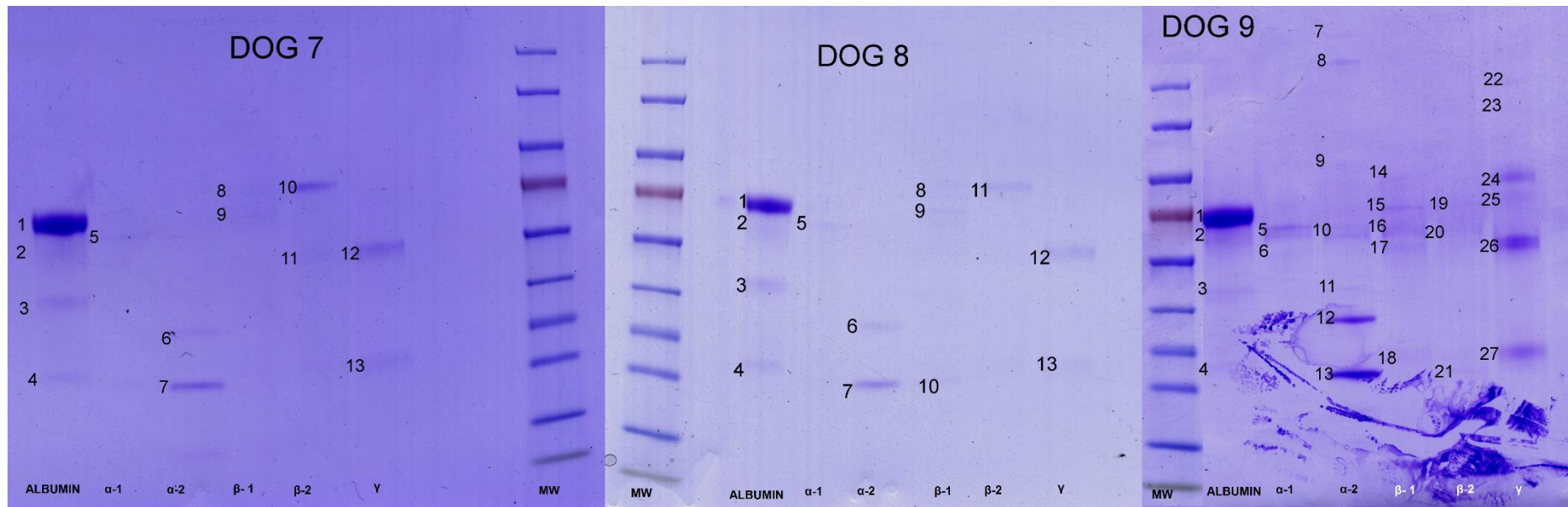


Figure 3-6: Image of two-dimensional serum protein electrophoresis gels from three dogs with high grade mast cell tumours. Each band and protein fraction has been labelled. Bands present in more than one gel were then selected to perform mass spectrometry. Dog 7 was a 9 year old female neutered crossbreed with a 25mm MCT on an extremity; dog 8, a 14 year old female neutered crossbreed with a 60mm tumour on the trunk and dog 9, an 8 year old male neutered Labrador retriever, with a 32mm tumour of the trunk. All dogs died due to progressive MCT. Excised bands submitted for mass spectrometer analysis: dog 7- bands 6,7 and 9; dog 9- bands 5,7,8,9,10,11,14,,24,26,27

Protein Fraction	Protein identified by mass spectrometry	Dog(s)	Band(s)	MOWSE Score(s)
α -1, α -2, α -2 β -1 β -1 γ	albumin	9 9 7 9 7 9	5, 8, 9, 10 6, 7 14 9 24	206, 113, 84, 77 107, 201 107 201 71
α -1	<i>α-1-antiproteinase-like^a</i>	9	5	102
α -1 α -2	<i>α-2-HS-glycoprotein^{a b}</i>	9	5, 10	72, 156
α -1	<i>kininogen-1 isoformX2</i>	9	5	115
α -1 α -2	serpin peptidase inhibitor, clade A ^a	9 9	5 10	105, 69
α -2	<i>α-2-macroglobulin^{a b}</i>	9	7, 8	80, 317
α -2	<i>angiotensinogen^{a b}</i>	9	10	94
α -2	<i>antithrombin-III isoform 1^{a b}</i>	9	10	240
α -2	<i>apolipoprotein A-I</i>	9, 7	10, 6	212, 1103
α -2	<i>fetuin-B^{a b}</i>	9	10	75
α -2	<i>glial fibrillary acidic protein isoformX2^b</i>	9	7, 8	105, 92
α -2	haptoglobin	7	7	342
α -2 β -1	<i>haemopexin</i>	7 7	6 9	145 145
α -2 β -1 γ	immunoglobulin gamma heavy chains	7 9 9	8 10 26	78 246, 131 802, 539, 385, 331
α -2	<i>inter-α-trypsin inhibitor heavy chain H1 isoform X2^{a b}</i>	9	7	221
α -2	<i>leucine-rich α-2-glycoprotein^{a b}</i>	9	10	73
α -2	<i>maltase-glucoamylase, intestinal^{a b}</i>	9	7	78
α -2	<i>plasma protease C1 inhibitor^{a b}</i>	9	9	113
α -2	<i>pregnancy zone protein^{a b}</i>	9	8	84
β -1	<i>C4b-binding protein α chain isoform X1^b</i>	9	14	88
β -1 γ	<i>complement C3</i>	9 7 9 9	14 8, 9 24 27	482 378, 594 819 76
β -1 γ	<i>complement C4-A^b</i>	9 9	14 24	84 203

β -2	<i>serotransferrin isoforms</i>	7	10, 11	1768, 166
γ		9	24, 26	1006, 73
γ	beta-2-glycoprotein 1 precursor ^a	9	26	86
γ	Immunoglobulin heavy chain variable region	9	26	195, 172, 161, 77
γ	immunoglobulin lambda light chain variable region ^a	9	27	182, 75
γ	<i>immunoglobulin lambda-like polypeptide 5-like</i>	9	27	126
γ	<i>putative V-set and immunoglobulin domain-containing-like protein</i> ^{a b}	9	26	89
γ	Immunoglobulin kappa chain variable region ^a	9	27	130
Γ	<i>uncharacterised protein</i>	9	27	194

Table 3-11: Proteins identified from the serum of dogs with high grade cutaneous mast cell tumours after separation using a method of two-dimensional electrophoresis. Italic text denotes that proteins predicted from known genomic sequences. ^a denotes proteins not identified in the serum of healthy control dogs, ^b denotes proteins not identified in the serum of dogs with low grade, cutaneous mast cell tumours. MOWSE= Mascot molecular weight search.

In total, 15 proteins were identified from the serum of dogs with MCT, which were not identified in the serum of normal dogs, both from the control cases in this study and also from healthy dogs from a previous report (Atherton *et al* ., 2013a). Of the proteins listed in table 3-11, α -1-antiproteinase-like and serpin peptidase inhibitor, clade A were considered to be different parts of the same protein and have been denoted as α -1-antiproteinase. The vast majority (10) of these, were found within the α -2 fraction. Eleven of the 15 were identified in HG MCT samples only (Table 3-12).

Protein (alternative names)	Protein Fraction	Function	Changes in disease
α -1-antiproteinase (serpin family A member 1, α -1 antitrypsin)	α -1	Serine protease inhibitor. Acute phase protein with negative feedback on the inflammatory response (Jain, Gautam and Naseem, 2011)	Serum marker of human cancer (Varela and Lopez Saez 1995)
α -2-HS-glycoprotein ^{a,b}	α -2	Endocytosis Brain development Formation of bone tissue	Serum marker of human breast cancer (Jae <i>et al.</i> , 2009)
α -2-macroglobulin ^{a,b}	α -2	Protease inhibitor Binds chymase released from mast cells (Raymond <i>et al.</i> , 2009) Acute phase protein with negative feedback on the inflammatory response (Jain, Gautam and Naseem, 2011) Protects IL-6 (Murata, Shimada and Yoshioka, 2004)	Identified in the serum of cats and dogs with lymphoma (Atherton <i>et al.</i> , 2013b; Gerou-Ferriani <i>et al.</i> , 2011)
angiotensinogen ^{a,b}	α -2	Cleaved by renin in response to hypotension	
antithrombin-III isoform 1 ^{a,b}	α -2	Serine protease inhibitor	
fetuin-B ^{a,b}	α -2	Cysteine protease inhibitor	
inter- α -trypsin inhibitor heavy chain H1 isoform X2 ^{a,b}	α -2	Implicated in multiple inflammatory diseases	H1 isoform is produced by mast cells and involved in malignant transformation in human pulmonary neoplasia (Bourguignon <i>et al.</i> , 1999).
maltase-glucoamylase, intestinal	α -2	Intestinal brush-border enzyme	

leucine-rich α -2-glycoprotein ^{a,b}	α -2	Expressed during granulocyte differentiation Protein to protein interaction Signal transduction Cell adhesion and development	Increased concentration in serum of humans with ovarian and lung cancer (Andersen <i>et al.</i> , 2010) Increased serum concentration in human pancreatic cancer independent of increases in acute phase proteins (Kakisaka <i>et al.</i> , 2007)
plasma protease C1 inhibitor ^{a,b}	α -2	Regulates complement activation Serine protease inhibitor Acute phase protein with negative feedback on the inflammatory response (Jain, Gautam and Naseem, 2011)	Increased serum concentrations in human cancer (Starcevic, Jelic-Ivanovic and Kalimanovska, 1991)
pregnancy zone protein ^{a,b}	α -2	Proteinase inhibitor	Increased serum concentrations in human prostatic carcinoma (Adeola <i>et al.</i> , 2015) Upregulated in human and canine osteoarthritis (Gharbi <i>et al.</i> , 2013)
β -2-glycoprotein 1 precursor (apolipoprotein H)	β -2	Contributes to innate immunity Lipoprotein metabolism Coagulation	
putative V-set and immunoglobulin domain-containing-like protein ^{a,b}	γ	Immunoglobulin-acquired immunity	
immunoglobulin lambda light chain variable region	γ	Immunoglobulin-acquired immunity	
Immunoglobulin kappa chain variable region	γ	Immunoglobulin-acquired immunity	

Table 3-12: Description of proteins identified in the serum of dogs with cutaneous mast cell tumours, not identified in the serum of healthy control dogs.

4 Discussion

The research undertaken in this thesis has identified a number of potential biomarkers for MCT. There are both biomarkers which could have clinical application in predicting the biological behaviour of a MCT as well as proteins which are not at present readily identifiable, but which may be relevant biomarkers when considering pathogenesis and/or treatment.

4.1 Neutrophil to lymphocyte ratio and albumin to globulin ratio

Within the studied population of 61 dogs, the NLR had good discriminatory ability in distinguishing between histopathological (Kiupel) grade of MCT. This has relevance as it may alter the investigative procedures required before treatment or may guide treatment decisions. The NLR utilises the role of inflammation in both initiating and propagating neoplastic change (Hanahan and Weinberg, 2011; Sideras and Kwekkeboom, 2014; Raposo *et al.* , 2015). A critical review of over 60 studies containing more than 37,000 human patients concluded that the NLR is useful in determining prognosis and disease stage for a variety of solid neoplasms (Guthrie *et al.* , 2013).

Given the complexity at both local and systemic levels, it is surprising that this ‘crude’ biomarker has shown utility in a wide range of neoplasms (Sideras and Kwekkeboom, 2014). The role of inflammation in any cancer is remarkably complex with both suppression and stimulation of very similar arms of the inflammatory response present within the tumour microenvironment (Raposo *et al.* , 2015). The use of the NLR in MCTs is further complicated given that mast cells are leucocytes, can create a marked inflammatory response (Blackwood *et al.* , 2012) and are involved in chemotaxis and interaction with both neutrophils and lymphocytes (Salamon *et al.* , 2005).

There is an indication for a biomarker which can be calculated pre-biopsy and has good discriminatory ability between LG and HG MCT to identify dogs which would benefit from a pre-treatment incisional or core biopsy to confirm

histopathological grade. In order to determine histopathological grade a biopsy is required. Typically, biopsy procedures other than a FNA are rarely performed on MCTs prior to surgery (Risselada, Mathews and Griffith, 2015). This may be due to concerns over increased complication rates due to mast cell degranulation or coagulopathies which can be present, financial considerations or that cytology is diagnostic in 96% of MCTs (Baker-Gabb, Hunt and France, 2003). In contrast, a complete blood count frequently forms part of a pre-anaesthetic assessment or general health profile in dogs with neoplasia and therefore NLR can often be attained with no morbidity to the dog or financial cost to the owner.

Current guidelines advise using the presence of factors associated with a more aggressive tumour as criteria for performing staging prior to surgery (Blackwood *et al.* , 2012; London and Thamm, 2012). The prevalence of regional metastasis is higher in tumours of a higher grade in this report and others (using the three-tiered grading system) (Krick *et al.* , 2009; Warland *et al.* , 2014). An objective biomarker which can aid in identification of HG tumours could be incorporated into this algorithm.

In human medicine, NLR is used to guide treatment decisions (Zou *et al.* , 2016) and this may also be applicable in canine MCTs. Current consensus guidelines for MCTs recommend larger surgical margins for higher grade tumours (using the three tiered grading system), although grade is often not known prior to surgery (Blackwood *et al.* , 2012). A discriminatory biomarker could therefore assist this decision making process. The NLR may also guide decisions such as the use of neo-adjuvant therapies, although objective data is currently lacking in this area in human medicine (Guthrie *et al.* , 2013).

NLR was maintained within the multivariable linear regression model, with each one point increase in NLR resulting in a two fold increase in the risk of having a HG MCTs when values were adjusted for age. In this population, there is an overlap in NLR values between LG and HG MCTs and therefore a perfect cut-off for NLR does not exist. We chose a value (5.67) which was sensitive for HG

disease to reduce misclassification of HG MCTs. Two HG MCTs (14%) were misclassified using the proposed NLR threshold value; one from a dog which had a HG MCT completely excised three months prior to inclusion, but at point of inclusion into the data set, a markedly enlarged inguinal lymph node was identified without local recurrence. Lymph node metastasis may therefore stimulate alternative inflammatory pathways to a primary cutaneous tumour, with a differing impact on the NLR. The other misclassified HG MCT had 'weak' KIT staining and a low Ki-67 score (0.45), indicating the possibility of LG biological behaviour, although the KIT staining pattern was type III, which has been related to an aggressive biological behaviour (Kiupel *et al.* , 2004; Scase *et al.* , 2006). Approximately half (twenty-two of forty-eight) of LG tumours were misclassified using the proposed NLR threshold value. Of the tumours where Ki-67 staining and KIT staining pattern was available, two of nine misclassified LG tumours had a high Ki-67 index (>1.8) and one of nine had a type III KIT staining pattern- both factors which can confer a higher biological grade (Kiupel *et al.* , 2004; Scase *et al.* , 2006), whilst three of eight of the correctly classified LG MCTs had a high Ki-67 index and one of eight had a type III KIT staining pattern. The impact of this misclassification is that additional diagnostic testing may be performed on cases with LG MCT with a NLR above the threshold value which may not be required. However, staging may still be appropriate for these cases as LG MCT have metastatic potential.

Age was maintained within the multivariable logistic regression model and an association between increasing age and more aggressive biological behaviour has previously been identified (Kiupel *et al.* , 2005).

Adjusting for other factors within the multivariable linear regression model, tumour size was not found to be associated with grade of MCT ($p=0.591$). There were several limitations to the measurement of tumour size, each possibly resulting in type two error. The longest diameter was used as this is the value which was most frequently reported in clinical notes, however, volumetric assessment as previously used in MCTs (Stanclift and Gilson, 2008) may be more

useful, but was unobtainable from available information. Method of measurement was not recorded as to whether it was estimated, measured with a caliper or whether imaging modalities were used. The longest diameter of metastatic lymph nodes was included, although a smaller proportion a lymph node structure may be expected to be comprised of neoplastic cells than a primary tumour.

There are conflicting reports regarding the validity of the World Health Organisation staging system for MCTs (Thamm, Mauldin and Vail, 1999; Kiupel *et al.*, 2005; Mullins *et al.*, 2006; Murphy *et al.*, 2006). Outcome variables were not assessed; however eight of nine dogs with multiple tumours had a LG MCT and there was a reduced probability of stage III tumours being HG than stage I or II disease on the univariate linear regression analysis (Table 3-4).

The importance of MCT location on biological behaviour is unclear. Four main locations, including head and neck, trunk, inguinal and perineal, or extremity have previously been used to evaluate the difference between sites (Kiupel *et al.*, 2005). Differing systems have been used in recent reports however (Donnelly *et al.*, 2013; Smith *et al.*, 2015) and a standard method of reporting MCT location to allow comparison between reports may be beneficial. Inguinal and perineal MCTs may have a worse prognosis in some studies (Sfiligoi *et al.*, 2005) but not others (Cahalane *et al.*, 2004). In this population, MCTs located on the trunk (designated 'other') were found to have the highest median NLR. Forty-five percent (five of eleven) of MCTs on the body were HG, compared to 23% (fourteen of sixty-two) of MCTs overall.

AGR was not useful in discriminating between high and LG MCTs in this population of 61 dogs. This biomarker was chosen given its use in human neoplasia (Azab *et al.*, 2013) and lack of additional cost or stress in its attainment given the frequency of biochemical analysis in this cohort of dogs. Albumin is an acute phase protein and it might be expected that its concentration would be lower in HG MCTs which are more likely to be

inflammatory. Its concentration was not different between LG and HG in this cohort.

Subsequent to this analysis, another study was conducted showing NLR not to be predictive of prognosis in a multivariable model for MCT (Skor *et al.* , 2017). In contrast, the neutrophil to eosinophil ratio was found to have prognostic significance. Using survival as an outcome variable for mast cell tumour is also problematic as the majority of dogs with MCT will not die as a result of their disease. The eosinophil concentration was not assessed in the population of dogs in this thesis, however, examination of whether the neutrophil to eosinophil ratio could be used to predict grade and comparison to the NLR would be a potential avenue for future research.

There were several limitations to the NLR and AGR research performed in this section. The case numbers presented are smaller than in thirty-two of thirty-four human reports collated in a review of the clinical use of the NLR (Guthrie *et al.* , 2013). The number of dogs with HG MCTs (fourteen) was particularly small, however the proportion of high and LG MCTs was similar to that previously reported (Kiupel *et al.* , 2011). Full staging procedures were not performed in every dog, so the incidence of metastatic disease, especially the involvement of local lymph nodes may be higher than reported. FNA of gross MCTs was performed at varying times prior to referral and also within our institution. There are no data available on the effect of FNA on haematological and biochemical parameters, but it is possible that a local inflammatory reaction to FNA may alter either NLR or AGR. Although NLR has been used as a prognostic marker in many human neoplastic conditions (Guthrie *et al.* , 2013), survival analysis was not performed because of the small number of cases, the variety of treatment modalities used, long survival times for LG MCTs and lack of complete follow up information which made the outcome of questionable scientific value. A larger study examining the prognosis or disease free intervals in a population of dogs with the same grade of MCT, treated in the same manner would be required to determine whether the NLR could be used as a stand-alone prognostic factor for MCTs.

4.2 Serum protein electrophoresis

Significant changes to the serum proteome of dogs with HG MCTs have been identified despite no overall increase in serum total protein concentrations. Changes in globulin fractions were found both with the affordable, accessible and available 1D-SPE and also 2D-PAGE protein separation and MS, using a previously described technique (Atherton *et al* ., 2013a).

1D-SPE identified an increase in the α -2 protein concentrations in HG MCTs compared to LG MCTs and control dogs. Increased α -2 concentrations are found in dogs with other neoplasms, including lymphoma and hepatocellular carcinoma (Atherton *et al* ., 2013b; Tappin *et al* ., 2011). This protein fraction contains acute phase proteins which increase in concentration in many inflammatory conditions, including MCT (Tappin *et al* . 2011; Chase *et al* ., 2012). Given the increased number of proteins identified in this fraction in HG MCT cases using MS, it is likely that this difference is attributable to changes in a number of proteins, although quantification of individual proteins was not possible.

Concentration of the β -1 fraction was increased in both HG and LG MCTs compared to control dogs. This fraction comprises transferrin, immunoglobulins and complement and is increased in many local and systemic neoplasms as well as both infectious and inflammatory conditions (Tappin *et al* ., 2011). No proteins were identified in the β -1 fraction of dogs with MCTs that were not also identified in the control population. This supports the hypothesis that differences in concentrations of particular fractions may be due mostly to changes in concentration of normal proteins rather than production of significant concentrations of novel or different proteins.

Changes in protein fractions detected in 1D-SPE are likely to result from a non-specific inflammatory response to MCT. It is possible that 1D-SPE will have a clinical utility in MCT since increased α -2 may suggest biological aggressiveness.

The pattern of 2D-PAGE protein bands was similar to that obtained by Atherton (2013a, b). The locations of complement proteins, kininogen and haptoglobin were similar amongst control dogs, LG and HG MCT groups and also to previous work analysing the serum of dogs with lymphoma. The fact that similar migration patterns were seen shows that this technique, when performed by different operators and using dogs with different tumour types is reproducible.

Despite this reproducibility, more proteins were found in control dogs in this analysis than in the control dogs examined by Atherton (2013a) when this technique was used for the first time. Nine proteins found in control dogs were the same in both studies, but Atherton identified two proteins not seen in this study's control population and conversely this thesis research identified 12 proteins not identified by Atherton. This variation may be due to the difference between brands of agarose gel used in the two studies and slightly different running conditions or to differences in the control population although both studies used donor controls. Many proteins, including albumin, were identified in multiple fractions. The separation technique is subject to experimental variation in how bands are excised from the unstained agarose gel and combined for the PAGE so some proteins can appear to be in the wrong fraction, creating additional bands on some gels or additional proteins co-migrating within bands. Other reasons for proteins being identified in more than one fraction include fragmentation of proteins during processing, leading to migration within a different band. Post-translational modification, different protein isoforms and streaming of proteins within the gel can also be causes of this identification in more than one fraction. Haptoglobin was identified in a dog from the control population. This was also identified in the α -2 fraction of a dog with lymphoma, but not previously in the serum of healthy dogs (Atherton *et al.* , 2013a,b). Despite its status as an acute phase protein, the identification of haptoglobin in the serum of a normal dog is not surprising as it is a constitutive protein (Murata, Shimada and Yoshioka, 2004).

Examination of the protein migration pattern found using PAGE, revealed that an increased number of unique bands were found in one of the HG MCT dogs (dog 9) which was the dog with shortest survival time. This can be seen as a limitation of the study as a large proportion of the protein identification data arose from one dog. Many of these proteins were within the α -2 and β -1 fractions, substantiating the 1D-SPE results. The other two HG MCT cases used for MS had longer survival times although both were euthanised due to MCT disease. These cases had fewer protein bands identified than the case with the shortest survival time. Cases were selected as having typical 1D-SPE results for their group and in retrospect it may have been more appropriate to this investigation to select cases with a more aggressive disease course.

After MS analysis, 16 proteins were identified within the serum of dogs with MCTs which were not identified in healthy control dogs. Four of these were acute phase proteins: α -1-antiproteinase, α -2-HS-glycoprotein, α -2-macroglobulin and plasma protease C1 inhibitor. One of these, α -1-antiproteinase, a protein found to increase the migration and invasion of human gastric cancer cells (Kwon *et al.* , 2014) was identified in the α -1 fraction. This protein, also known as serpin peptidase inhibitor, clade A was found in band 4 from dog 5. This protein has also been identified in the serum of dogs with lymphoma, using the same method and was in a similarly located band in the same protein fraction (Atherton *et al.* , 2013b). Although a similarly located band was identified within the control population this protein was not identified within it. Five of the 16 MCT specific proteins were proteinase inhibitors: α -1-inhibitor, α -2-macroglobulin, antithrombin III, plasma protease C1 inhibitor and pregnancy zone protein. Proteinase inhibitors may be synthesised in response to an inflammatory state, but have also been associated with tumour pathogenesis, possibly by stimulating the production of matrix metalloproteases (Sueoka *et al.* , 2005). α -2 macroglobulin binds chymase released by mast cells (Raymond *et al.* , 2009) and provides negative feedback to an inflammatory response (Jain, Gautam and Naseem, 2011). α -2 macroglobulin has also been identified in the serum of dogs and cats with lymphoma (Gerou-Ferriani *et al.* , 2011, Atherton *et*

et al., 2013b;). Canine specific enzyme linked immunosorbent assays are available and identification in a larger cohort of dogs with MCT is indicated. Further exploration of the role of protease inhibitors in MCT is warranted to determine their role in this cancer.

α -1-antiproteinase, α -2-HS-glycoprotein, leucine-rich α -2-glycoprotein, plasma protease C1 inhibitor and pregnancy zone protein have been identified in the serum of human patients with various neoplastic conditions and are used as biomarkers (Starcevic *et al.*, 1991; Varela and Lopez Saez 1995; Kakisaka *et al.*, 2007; Jae *et al.*, 2009; Andersen *et al.*, 2010; Adeola *et al.*, 2015). Identification and quantification of these proteins in a wider cohort of MCT and control animals is indicated to assess their potential use as biomarkers.

Inter- α -trypsin inhibitor heavy chain H1 isoform is one of three precursor proteins, typically produced in hepatocytes, which covalently bind to form inter- α -trypsin inhibitor. The H1 isoform has been identified and may also be synthesised within mast cells and neoplastic pulmonary squamous cells (Bourguignon *et al.*, 1999). Although a specific role has not been identified, inter- α -trypsin inhibitor contributes to malignant transformation (Bourguignon *et al.*, 1999) and may be involved in the pathogenesis of MCT. This protein was identified within the α -2 fraction (Figure 3-6, Dog 9 Band 7) with a band not identified in LG MCT or control samples and was the highest MOWSE score protein identified within this band. Additional 2D-PAGE and MS, immunohistochemistry or commercially available canine specific enzyme linked immunosorbent assays may therefore be warranted to identify this protein in further serum or tissue samples from dogs with HG MCT.

This technique of proteomic evaluation identified a large number of proteins with confidence and within expected protein bands. The MOWSE score cut-off employed may result in not identifying some proteins present in the samples, but was used to ensure confidence in the results.

The limitations of this study are the small sample sizes which could result in both type one and two error. Not every protein band identified from the 2D-PAGE was submitted for MS analysis. Doing so may have allowed identification of the same protein in multiple dogs within the same group, increasing the reliability of the published data. Additional proteins may also have been identified as a result. The HG MCT group contained tumours which were significantly larger and more dogs with a higher tumour stage than the LG MCT, therefore proteomic changes may be a result of increasing tumour size and stage alone. However, the dog with most protein bands identified (dog 9), had a tumour below the median tumour size for HG MCT and had stage I disease. Additionally, the LG MCT dog with stage II disease (dog 5) had the fewest proteins bands (9).

The control group had a significantly younger age than the LG and HG groups. Younger dogs are less likely to have disease and therefore some of the proteins identified may be as a consequence of comorbidities in the LG MCT and HG MCT populations. Significant comorbidities resulted in exclusion from the study population; any comorbidities affecting the results would therefore have been subclinical. Also, increasing age was not found to influence 1D-SPE results in healthy dogs (Tappin *et al.* , 2011). Ideally, age matched controls would be used in any future research.

Finally, it is not known whether the proteomic alterations identified are released from the MCT or transcribed as part of an associated inflammatory reaction. The serum proteins identified in the serum of dogs with HG MCT were not identified in a previous proteomic analysis of MCT tissue (Schlieben *et al.* , 2012), suggesting that they may be an inflammatory response. If the nature of an inflammatory response was specific however, it would not preclude use as a biomarker.

5 Conclusions

5.1 Neutrophil to lymphocyte ratio

Within this population of dogs, the NLR was a good discriminatory tool to select HG MCTs prior to histopathological assessment. The ratio was found to be more useful than either of its constituent parts used alone. A cut-off value of 5.67 was selected which was highly sensitive for detection of HG tumours, ensuring that dogs with a disease with an expected aggressive clinical course were not undertreated. This biomarker may have a clinical application, such as deciding on the usefulness or extent of staging or optimal treatment regimen, including planned surgical margins. The limitations of this study however, mean that further validation of the threshold value is recommended in further prospective case series prior to widespread clinical use. Analysis of its utility as a prognostic marker could also be considered. The cut-off value chosen here did result in many low grade tumours being incorrectly classified. One area worthy of further research would be a comparison of the clinical course and prognosis of those LG MCT with a NLR above and below the cut-off value. NLR has previously been identified as a useful tool to differentiate between benign and malignant soft tissue tumours (Macfarlane *et al* ., 2015). As in human neoplasia, it is likely that this biomarker could be useful in other difficult clinical situations, such as determining prognosis within a specific subset of dogs with lymphoma.

5.2 Serum protein electrophoresis

Significant changes were identified in the serum proteome of dogs with HG MCTs. Using 1D-SPE, these included an increase in the α -2 and β -1 fractions when compared to healthy controls. The concentration of α -2 proteins was also increased in dogs with HG MCT compared to those with LG MCTs. For this finding to be clinically significant 1D-SPE would have to be compared with a larger number of cases.

Using 1D-SPE combined with PAGE, 16 proteins mainly from the α -2 fraction were identified in the serum of dogs with MCTs which were not identified in healthy control dogs. Identified changes may reflect an inflammatory response to the tumour or may be produced by the MCT and contribute to a more aggressive phenotype. Many of these proteins, such as α -1-antiproteinase, α -2-HS-glycoprotein, leucine-rich α -2-glycoprotein, plasma protease C1 inhibitor and pregnancy zone protein have been identified in the serum of human patients with various neoplastic conditions and are used as biomarkers for these. Further investigation, initially focussed on identifying these proteins from the same protein band in a larger group of dogs with MCTs would be useful. If proteins were identified in other dogs with MCTs, then future work should be directed at attempting to quantify these proteins. Inter- α -trypsin inhibitor heavy chain H1 looks like the more promising target for further investigation, due to its production within mast cells (Bourguignon *et al.* , 1999).

It is hoped that results of this investigation will stimulate further research to identify and quantify specific proteins in larger cohorts of dogs with MCTs. This may identify biomarkers which could be used to prognosticate, guide treatment decisions, identify therapeutic targets or improve understanding of the pathogenesis of MCTs.

The method of protein identification and separation used has proved to be useful to identify potential biomarkers in canine MCTs and also in lymphoma. It is encouraging that two independent studies have identified the same proteins in bands at the same location on 2D-PAGE gels in both cancers and in normal dogs. This technique could therefore be used to identify biomarkers in other canine tumour types. In particular, much research has been done on identifying biomarkers in canine mammary cancer (Pena *et al.* , 2014; Estrela-Lima *et al.* , 2016; Raposo *et al.* , 2016) and further identification of serum biomarkers may be a powerful tool for this tumour type.

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Appendices

Breed	Sex	Neuter	Age	MCT Grade	Neutrophil	Lymphocyte	NLR	Albumin	Globulin	AGR	Clinical signs	Size	Location	WHO Stage	Lymph Node Metastasis
Boxer	F	N	7	Low	3.20	2.25	1.42	30	36	0.83	No	9	Multiple	3	No
Beagle	F	N	6	Low	3.64	1.38	2.64	29	29	1	No	58	Multiple	3	No
Labrador Retriever	M	N	9	Low	6.61	2.32	2.85	27	35	0.77	No	40	Multiple	3	No
West Highland White Terrier	M	E	4	Low	6.68	2.26	2.95	31	34	0.91	No		Head/Neck	1	No
Labrador Retriever	M	N	4	Low	5.97	1.99	3	33	30	1.1	No	16	Extremity	1	No
Boxer	F	N	3	Low	6.08	2.03	3	33	33	1	No	8	Head/Neck	1	No
Crossbreed	M	N	12	Low	6.64	2.03	3.27	35	32	1.09	Yes	10	Extremity	1	No
Boxer	M	E	5	Low	5.71	1.68	3.4	33	33	1	No	25	Multiple	3	No
Boxer	M	N	5	Low	3.71	1.09	3.4	29	37	0.78	No	6	Extremity	1	No
Golden Retriever	M	N	11	Low	4.18	1.21	3.45	28	29	0.97	No	25	Extremity	1	No
Border Collie	M	N	1	Low	12.01	3.25	3.7	29	36	0.81	No	6	Head/Neck	1	No
Staffordshire Bull Terrier	F	N	10	Low	4.26	1.15	3.7	31	35	0.89	No	22	Head/Neck	1	No
Labrador Retriever	F	E	5	Low	8.47	2.17	3.89	30	36	0.83	No	10	Head/Neck	1	No
Golden Retriever	F	N	6	Low	7.65	1.86	4.12	31	35	0.89	No	20	Head/Neck	1	No

Boxer	M	N	5	Low	6.42	1.49	4.3	27	38	0.71	No	21	Head/Neck	2	Yes
Golden Retriever	F	N	10	Low	6.88	1.59	4.33	32	33	0.97	No	40	Multiple	3	No
Labrador Retriever	M	E	7	Low	7.18	1.59	4.53	34	29	1.17	No	9	Head/Neck	1	No
Crossbreed	F	E	6	Low	6.36	1.38	4.63	33	34	0.97	No	20	Extremity	1	No
Miniature Schnauzer	M	N	4	Low	5.51	1.18	4.69	36	38	0.95	Yes	20	Extremity	1	No
Labrador Retriever	F	N	11	Low	4.44	0.92	4.8	32	30	1.07	No	12	Other	1	No
Staffordshire Bull Terrier	M	E	9	Low	3.76	0.77	4.87	32	28	1.14	No	40	Multiple	3	No
Crossbreed	F	N	8	Low	6.41	1.25	5.13	32	36	0.89	No	12	Head/Neck	1	No
Golden Retriever	F	N	6	Low	6.33	1.19	5.33	29	31	0.94	No	8	Extremity	1	No
Crossbreed	F	N	11	Low	6.79	1.22	5.57	32	31	1.03	No	15	Extremity	1	No
Pyrenean Mountain Dog	F	N	5	Low	6.86	1.22	5.62	33	31	1.06	No		Extremity	2	Yes
Boxer	M	N	7	Low	5.55	0.98	5.64	31	30	1.03	No	45	Multiple	3	No
Labrador Retriever	F	E	2	Low	9.94	1.72	5.79	30	29	1.03	No	8	Head/Neck	1	No
Labrador Retriever	F	N	7	Low	7.77	1.31	5.92	33	30	1.1	No	20	Head/Neck	1	No
Vizsla	M	E	2	Low	7.30	1.20	6.08	32	43	0.74	No	40	Extremity	1	No
Springer Spaniel	F	N	11	Low	6.29	1.00	6.31	32	40	0.8	No	20	Head/Neck	1	No
Boxer	M	N	7	Low	8.93	1.37	6.5	31	36	0.86	No	15	Head/Neck	1	No

Crossbreed	F	N	8	Low	10.35	1.59	6.5	32	40	0.8	Yes	53	Extremity	2	Yes
Boxer	M	N	4	Low	4.80	0.73	6.58	28	36	0.78	No	30	Extremity	1	No
Boxer	M	N	9	Low	4.80	0.73	6.58	28	36	0.78	No		Extremity	1	No
Boxer	M	E	5	Low	6.11	0.93	6.58	31	31	1	No	25	Extremity	1	No
Labrador Retriever	M	N	9	Low	5.92	0.90	6.58	28	34	0.82	No	30	Extremity	1	No
Labrador Retriever	M	E	6	Low	7.82	1.17	6.68	34	29	1.17	No	9	Extremity	1	No
Labrador Retriever	F	E	9	Low	6.99	1.04	6.75	32	30	1.07	No	20	Other	1	No
Labrador Retriever	F	N	7	Low	8.42	1.25	6.75	32	28	1.14	No	45	Extremity	2	Yes
Crossbreed	M	N	8	Low	8.47	1.26	6.75	29	29	1	No	43	Extremity	2	Yes
Boxer	M	N	4	Low	8.69	1.27	6.83	32	37	0.86	No	32	Head/Neck	1	No
Springer Spaniel	M	N	9	Low	6.16	0.88	7	32	34	0.94	No	80	Extremity	1	No
Golden Retriever	F	N	6	Low	6.79	0.95	7.16	31	35	0.89	Yes	24	Other	1	No
Boxer	F	N	4	Low	5.31	0.65	8.2	34	38	0.89	No	50	Multiple	3	No
Anatolian Shepherd	M	N	8	Low	6.32	0.74	8.5	33	31	1.06	No	30	Other	1	No
Labrador Retriever	F	E	7	Low	8.26	0.96	8.59	31	33	0.94	No	25	Other	1	No
Golden Retriever	M	E	9	Low	8.81	1.02	8.6	30	32	0.94	No	35	Extremity	2	Yes
Boxer	M	E	8	Low	6.54	0.74	8.89	32	35	0.91	No	10	Extremity	1	No

Labrador Retriever	M	N	7	High	5.13	1.34	3.83	35	32	1.09	No	13	Head/Neck	1	No
Crossbreed	M	N	12	High	3.62	0.78	4.63	32	34	0.94	No	40	Other	2	Yes
Labrador Retriever	F	N	10	High	5.89	1.04	5.69				Yes	20	Extremity	1	No
Labrador Retriever	M	N	13	High	7.66	1.15	6.67	29	34	0.85	Yes	20	Head/Neck	2	Yes
Labrador Retriever	M	N	10	High	6.91	0.89	7.8	21	27	0.78	No	40	Other	1	No
Jack Russell Terrier	M	E	9	High	8.32	1.02	8.2	25	32	0.78	No	90	Extremity	2	Yes
Crossbreed	F	N	14	High	9.58	1.10	8.7	33	33	1	No	60	Extremity	2	Yes
Staffordshire Bull Terrier	M	E	5	High	7.13	0.78	9.15	33	34	0.97	No	40	Other	1	No
Labrador Retriever	M	N	8	High	8.98	0.86	10.5	27	45	0.6	No	32	Other	1	No
Crossbreed	M	N	6	High	8.50	0.78	10.91	34	36	0.94	No	12	Multiple	3	No
Labrador Retriever	M	E	7	High	13.36	0.78	17.21	29	31	0.94	Yes	140	Other	2	Yes
Lhasa Apso	F	E	10	High	15.18	0.85	17.8	27	41	0.66	Yes	30	Head/Neck	1	No
Crossbreed	F	N	9	High	10.34	0.36	28.63	36	42	0.86	No	25	Extremity	1	No
Labrador Retriever	M	E	12	High	5.96	0.21	29.07	26	36	0.72	No	85	Head/Neck	2	Yes

Appendix 1: Information of all dogs which met inclusion criteria. M=male, F=female, E=entire, N=neutered, Neutrophil=neutrophil concentration ($\times 10^9/\text{l}$), Lymphocyte=lymphocyte concentration ($\times 10^9/\text{l}$), Albumin=albumin concentration (g/l), Globulin=globulin (g/l), Size in millimetres.